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Original Article

Deglycosylation of PD-L1 by 2-deoxyglucose reverses PARP inhibitor-induced immunosuppression in triple-negative breast cancer

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Abstract: Triple-negative breast cancer (TNBC), the most difficult-to-treat breast cancer subtype, lacks well-defined molecular targets. TNBC has increased programmed death-ligand 1 (PD-L1) expression, and its immunosuppressive nature makes it suitable for immune checkpoint blockade therapy. However, the response rate of TNBC to anti-PD-L1 or anti-programmed cell death protein 1 (PD-1) therapy remains unsatisfactory, as only 10-20% of TNBC patients have a partial response. Glycosylated PD-L1, the functional form of PD-L1, is required for PD-L1-PD-1 interaction. TNBC cells have significantly higher levels of glycosylated PD-L1 than non-TNBC cells do. In a screening of glucose analogs to block PD-L1 glycosylation, we found that 2-deoxyglucose (2-DG) can act as a glucose analog to decrease PD-L1 glycosylation. Because PARP inhibition upregulates PD-L1, 2-DG reduced PARP inhibition-mediated expression of glycosylated PD-L1. The combination of PARP inhibition and 2-DG had potent anti-tumor activity. Together, our results provide a strong rationale for investigating the targeting of PD-L1 glycosylation in TNBC further.

Keywords: 2-deoxyglucose, 2-DG, glycosylation, PD-L1, PD-1, immunosuppression, PARP inhibitor, triple-negative breast cancer

Introduction

Compared with other breast cancer subtypes, triple-negative breast cancer (TNBC), which lacks estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) overexpression, is more aggressive and is associated with higher rates of relapse and mortality. TNBC's heterogeneity and lack of well-defined molecular targets pose treatment challenges; in particular, TNBC has a short-lived response to systemic chemotherapy, the standard of care, and invariably develops resistance to such treatment. Thus, novel effective therapies for TNBC are urgently needed.

Immunotherapy is emerging as a promising cancer therapy including breast cancer, especially TNBC. TNBC has higher genomic instability and higher mutation rates, which can lead to the production of neoantigens and increased immunogenicity [1, 2]. TNBC also has increased expression of the programmed death-ligand 1 (PD-L1) in the tumor microenvironment [3], making the disease an ideal candidate for immune checkpoint blockade therapy. The programmed cell death protein 1 (PD-1)/PD-L1 inhibitory pathway can silence the immune system by increasing the expression of PD-L1 on the tumor cell surface [4]. Anti-PD-L1 or anti-PD-1 antibodies can restore T-cell function and increase the efficacy of standard chemotherapy

for TNBC. The impressive and durable clinical responses achieved with checkpoint blockade immunotherapy resulted in the U.S. Food and Drug Administration's approval of ipilimumab, nivolumab, pembrolizumab, and atezolizumab for the treatment of multiple cancer types, including melanoma, Hodgkin lymphoma, lung cancer, and bladder cancer [5-8]. However, the therapeutic efficacy of anti-PD-L1 or anti-PD-1 antibody in TNBC was not satisfactory. Post-translational modifications such as glycosylation control the critical biological activity of proteins and occur on approximately two-thirds of all proteins [9]. Glycosylated PD-L1, the functional form of PD-L1, interacts with PD-1 to suppress effector T cell activity in the tumor microenvironment [10-12]. In TNBC, PD-L1 is heavily glycosylated, making glycosylated PD-L1 an ideal therapeutic target in TNBC [13].

Poly (ADP-ribose) polymerase (PARP) inhibition (PARPi), by inducing synthetic lethality, is an effective therapeutic strategy against tumors associated with germline mutations in double-strand DNA repair genes [14]. PARPi has achieved promising results in patients with BRCA-mutated breast cancer in clinical trials. Olaparib was the first PARP inhibitor to show a significant progression-free survival benefit over standard therapy in a randomized phase III trial in patients with BRCA-mutated, HER2-negative metastatic breast cancer [15]. Another PARP inhibitor, talazoparib, achieved similar results in the EMBRACA study, a large phase III randomized trial in patients with germline BRCA1 or BRCA2 mutation [16]. Thus far, combining PARPi with other targeted therapy, immunotherapy, or chemotherapy is a new strategy in clinical trials. In a previous study, we found that PARPi treatment upregulates the PD-L1 expression of TNBC cells [17], and thus might reduce the response rate of PARPi. The above results suggest that combining PARPi and immunotherapy would be effective against TNBC. Since TNBC has heavily glycosylated PD-L1, which is the functional form of PD-L1. The purpose of this study was to explore the deglycosylation agents which have the potential for combination with PARPi in the treatment of TNBC.

Materials and methods

Cell lines

All cell lines (MB468, BT549, HCC1806 and MB231) were obtained from ATCC (Manassas, VA) and were independently validated by short tandem repeat DNA fingerprinting at MD Anderson Cancer Center. Cells were grown in Dulbecco's modified Eagle's medium/F12 medium or RPMI 1640 medium supplemented with 10% fetal bovine serum. PD-L1-stable transfectants in MDA-MB-231 and BT549 cells were selected using puromycin (InvivoGen, San Diego, CA) and have been described previously [17].

Antibodies and agents

2-deoxyglucose (2-DG) was purchased from Sigma-Aldrich (St. Louis, MO; cat. #D8375). Biosciences (Lincoln, NE; cat. #926-08946). The PARPi (Olaparib and Talazoparib) and the anti-PD-L1 antibody (cat. #13684) were purchased from Cell Signaling Technology (Danvers, MA), and α-tubulin (cat. #B-5-1-2) was obtained from Sigma-Aldrich. Olaparib and talazoparib were purchased from Selleckchem (Houston, TX). Cycloheximide was purchased from Sigma-Aldrich. The human anti-CD274 (B7-H1, PD-L1) antibody for the T-cell killing assay was from BioLegend (San Diego, CA; cat. #329709).

Detection of cell-surface PD-L1

For the detection of cell-surface PD-L1, cells were suspended in 100 μL of cell staining buffer (BioLegend; cat. #420201) and incubated with the allophycocyanin (APC)-conjugated anti-human PD-L1 antibody (BioLegend; cat. #329-708) at room temperature for 30 minutes. The stained cells were washed in the staining buffer and then analyzed using a flow cytometer (BD Biosciences, San Jose, CA).

PD-L1/PD-1 interaction assay

Cells were incubated with 5 mg/mL recombinant human PD-1 Fc chimera protein (R&D Systems, Minneapolis, MN; cat. #1086-PD-050) at room temperature for 60 minutes. The cells were washed with staining buffer and then incubated with anti-human Alexa Fluor 488-conjugated antibody (Thermo Fisher Scientific, Waltham, MA) at room temperature for 30 minutes. The cells were then washed in staining buffer and then analyzed by fluorescence-activated cell sorting (FACS). The FACS data were analyzed using the FlowJo software program (Tree Star, Inc., Ashland, OR); the cut-off value for the relative positive percentage was the median of the maximum signal. For monitoring dynamic PD-1 protein binding on the live cell surface, PD-L1-expressing BT549 cells were

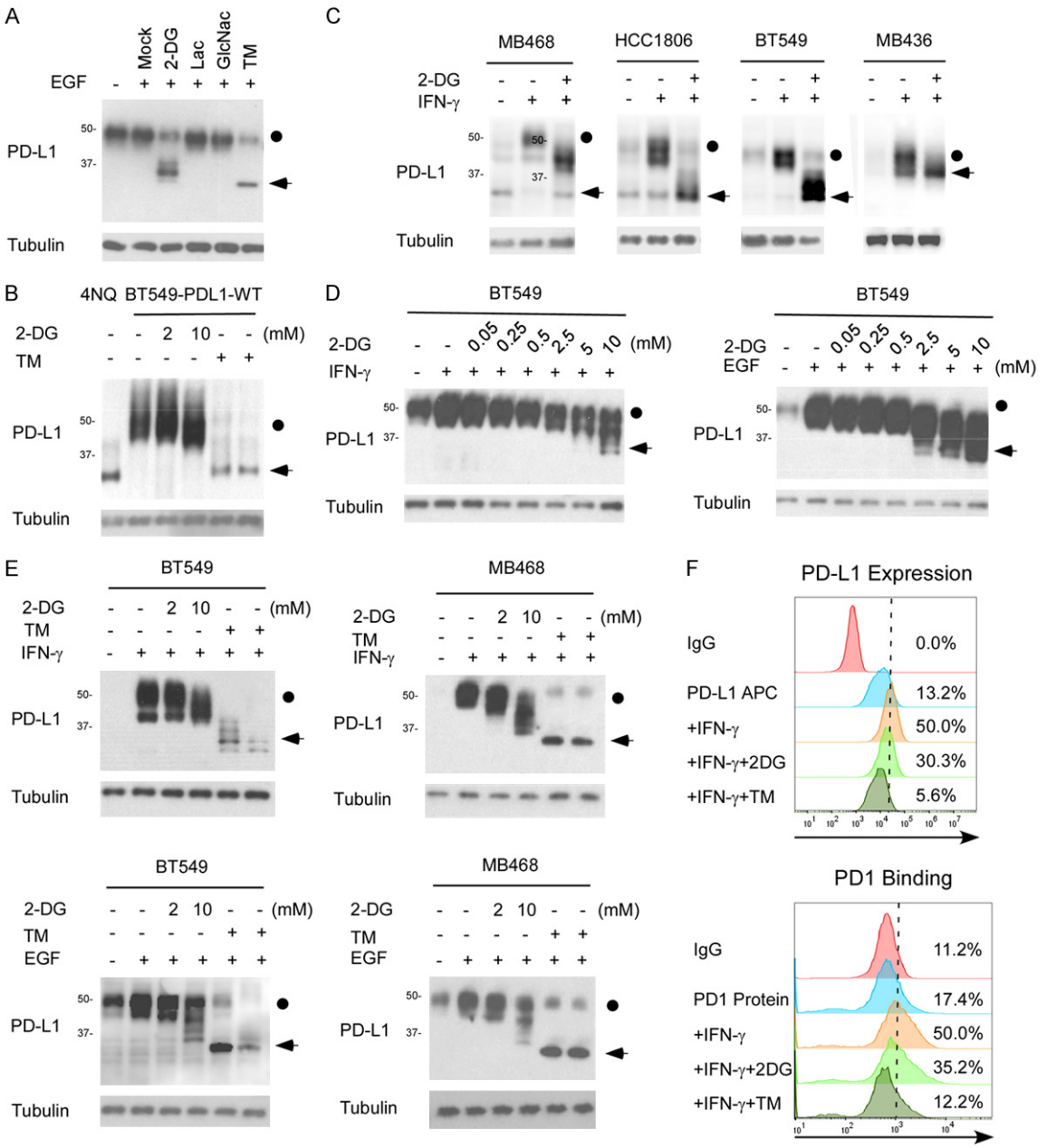

Figure 1. 2-DG deglycosylates PD-L1 in TNBC. A. Western blot analysis of PD-L1 protein expression in MDA-MB-231 cells treated with 2-DG, lactacystin (Lac), N-acetylglucosamine (GlcNac), or tunicamycin (TM). The dot indicates the glycosylated PD-L1 and the arrow indicates the deglycosyalted PD-L1. B. PDL1-overexpressing BT549 cells were treated with 2 or 10 mmol/L 2DG or with 1 μgml^{-1} TM for 12 hours and then subjected to immunoblotting with antibodies against PD-L1. BT549-4NQ cells were included as a negative control. C. Western blot analysis of PD-L1 protein expression in MB468, HCC1806, and BT549 cells treated with IFN-γ with or without 2-DG. D. BT549 cells were treated with indicated concentrations of 2-DG and IFN-γ or EGF for 12 hours, and then PD-L1 protein expression was determined by Western blot analysis. E. Western blot analysis of PD-L1 protein expression in MB468 and BT549 cells treated with 2 or 10 mmol/L 2-DG in combination with IFN-γ or EGF. TM treatment was used as the positive deglycosylation control. F. PD-L1 expression and PD-1 binding on the surface of BT549 cells were analyzed with FACS. The cells were treated with 10 mmol/L 2-DG, 1 μgml^{-1} TM, or 1 μgml^{-1} IFN-γ for 12 hours.

incubated with Alexa Fluor 488-conjugated PD-1 Fc protein and images of the cells were captured every hour using an IncuCyte ZOOM microscope (Essen BioScience, Ann Arbor, MI).

T cell-mediated tumor cell killing assay

MDA-MB-231 cells were seeded into a 24-well plate and NucLight Red (nuclear-labeled red

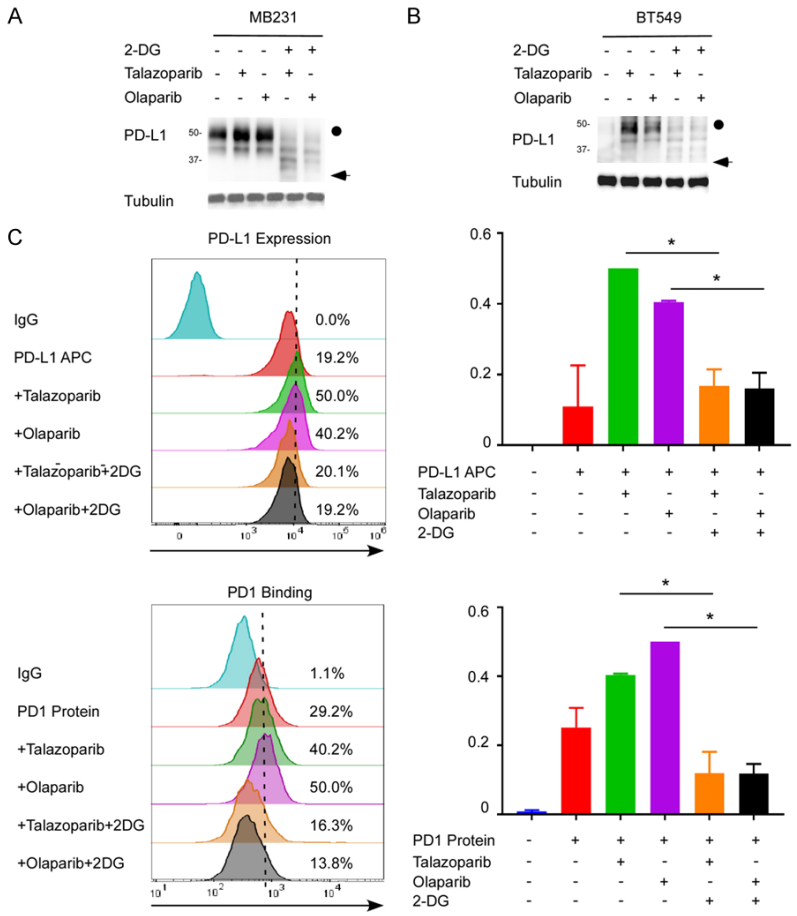

Figure 2. 2-DG deglycosylates PARPi-induced PD-L1 protein in TNBC. A. Western blot analysis of the PD-L1 protein expression of MDA-MB-231 cells treated with 2-DG and/or PARPi (talazoparib or olaparib). B. Western blot analysis of the PD-L1 protein expression of BT549 cells treated with 2-DG and/or PARPi (talazoparib or olaparib). C. PD-L1 expression and PD-1 binding on the surface of MDA-MB-231 cells treated with 2-DG and/or PARPi were analyzed with FACS. **P*<0.05.

fluorescent protein)-expressing MDA-MB-231 cells (Essen BioScience; cat. #4457) were seeded into a 96-well plate. Human peripheral blood mononuclear cells (PBMCs) obtained from Stemcell Technologies (Vancouver, Canada; cat. #70025) were activated with 100 ng/mL anti-CD3 antibody, 100 ng/mL anti-CD28 antibody, and 10 ng/mL interleukin 2 (BioLegend; cat. #317303, #302913, and #589102, respectively) and then cocultured with MDA-MB-231 cells or NucLight Red-expressing MDA-MB-231 cells at a 15:1 ratio with treatment of PARPi (olaparib or talazoparib) and/or 2-DG for 48 hours. The NucLight Red-expressing MDA-MB-231 cells were cocultured with fluorescence caspase-3/7 substrate (Essen BioScience; cat. #4440).

Statistical analysis

Statistical analyses were performed using the SPSS Statistics 20.0 software program (SPSS, Chicago, IL). The Student t-test was used for experimental data. *P* values <0.05 were considered statistically significant.

Results

2-DG deglycosylates PD-L1 protein

2-DG treatment led to a band shift of PD-L1 from ~45 kDa to 33 kDa (**Figure 1A**, lane 3; **Figure 1B**, lanes 2 vs 4), suggesting that 2-DG downregulates the glycosylation of PD-L1. While Lactose and N-acetylglucosamine didn't observe the phenomenon. According to our previous experience [10], the 45-kDa PD-L1 is the glycosylated form of PD-L1 (**Figure 1A**, lane 1), and the 33-kDa PD-L1 is the non-glycosylated form of the protein (**Figure 1A**, lane 6; **Figure 1B**, lane 1, 5, 6). Similar effects were observed in other cell lines, including MB468, HCC1806, and BT549 (**Figure 1C**).

To investigate whether 2-DG downregulates the glycosylation of PD-L1 induced by interferon-gamma (IFN-γ) or epithelial growth factor (EGF), we pretreated BT-549 and MB468 cells with IFN-γ and EGF before the addition of 2-DG. That IFN-γ mediates the transcriptional regulation of PD-L1 via STAT or NF-κB is well established. EGF did not influence PD-L1 mRNA expression but enhanced PD-L1 glycosylation through the inactivation of GSK3β, which mediated PD-L1 degradation [10]. Similarly, 2-DG reduced the PD-L1 glycosylation induced by IFN-γ and EGF (**Figure 1E**). Treating cells with the indicated concentrations of 2-DG reduced PD-L1 glycosylation in a dose-dependent manner (**Figure 1D**). PD-L1 expressed on the surface of cancer cells exerts immunosuppressive effects by

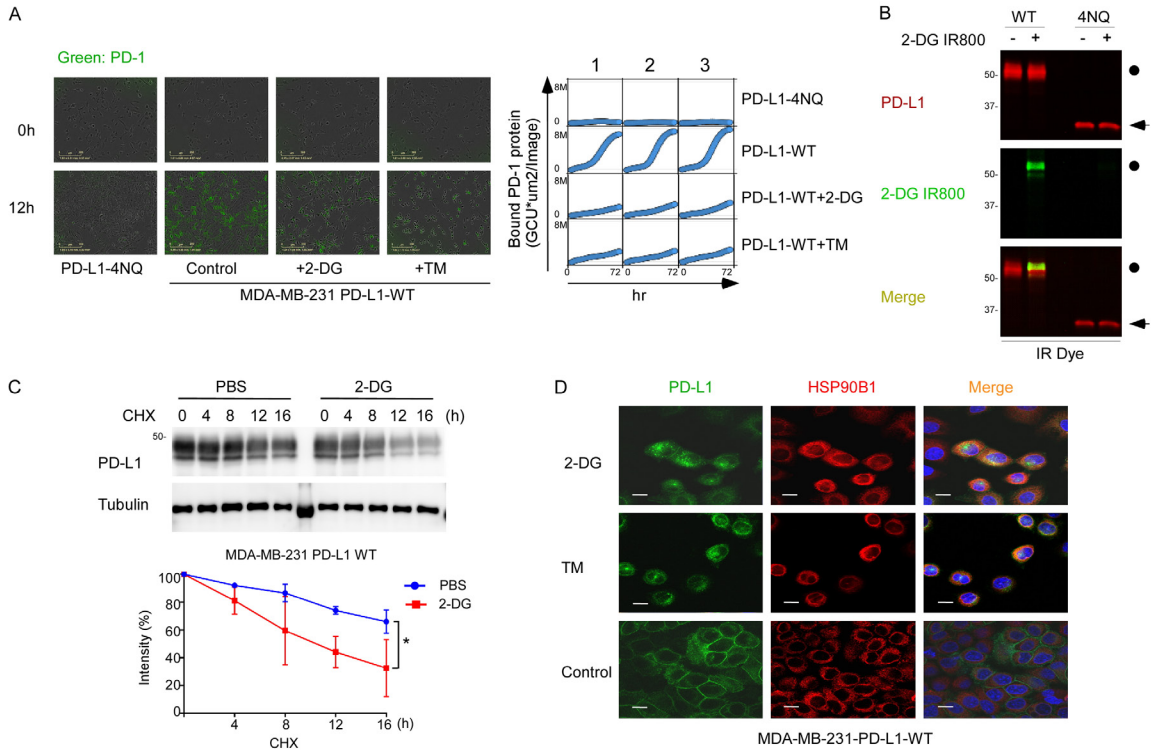

Figure 3. 2-DG decreases PD-L1 translocation and stabilization. A. Left, The quantitative binding of PD-1 Fc protein on PD-L1-overexpressing MDA-MB-231 cells was assessed at the indicated times. Cells were treated with 10 mmol/L 2-DG, 1 μgml^{-1} tunicamycin (TM), or 10 μmol/L olaparib. Right, Images of PD1 Fc protein on PD-L1-overexpressing MDA-MB-231 cells from 0 to 72 hours. B. Western blot analysis of PD-L1 protein expression in PD-L1-overexpressing MDA-MB-231 cells and MDA-MB-231-4NQ cells treated with 2 mmol/L 2-DG IR800. C. Western blot analysis of PD-L1 protein expression in PD-L1-overexpressing MDA-MB-231 cells. Cells were treated with 20 mM cycloheximide (CHX) with or without 2 mmol/L 2-DG at the indicated times. The intensity of PD-L1 protein expression was quantified using a densitometer. *P<0.05. D. Confocal microscopy image showing HSP90B1 and PD-L1 expression in PD-L1-overexpressing MDA-MB-231 cells after treatment with 2-DG. Scale bar, 20 mm.

binding with a PD-1 receptor on activated T cells. To determine whether 2-DG affects cell-surface PD-L1 expression levels, we treated IFN-γ-pretreated BT549 cells with 2-DG and tunicamycin. Cell-surface PD-L1 expression was assessed using an APC-conjugated PD-L1 antibody, and cells were analyzed with FACS. The cell-surface PD-L1 expression level was significantly reduced after treatment with 2-DG and tunicamycin (**Figure 1F**). To investigate the functional significance of PD-L1's downregulation by 2-DG, we assessed whether 2-DG alters the interaction between PD-1 and PD-L1 and found that 2-DG significantly reduced PD-1 binding levels in BT549 cells (**Figure 1F**). Together, these results suggest that 2-DG downregulates the glycosylation of both endogenous and cytokine-induced PD-L1.

2-DG deglycosylates PARPi-induced PD-L1 protein

Our western blot analysis revealed that PARPi consistently enhanced PD-L1 expression in MDA-MB-231 and BT549 cells which were consistent with our previous result [17]. To validate whether 2-DG reduces the PARPi-induced upregulation of PD-L1, we treated MDA-MB-231 and BT549 cells with 2-DG and/or PARP inhibitors. 2-DG significantly reduced not only PARPi-induced PD-L1 expression but also the basal level of PD-L1 (**Figure 2A**, **2B**). Similarly, the cell-surface PD-L1 expression levels were significantly decreased after 2-DG treatment. As a result, 2-DG also reduced the binding of PD-1 to PD-L1 on the cell surface (**Figure 2C**). Together, these results indicate that 2-DG deglycosylates PARPi-induced PD-L1 protein in TNBC.

2-DG downregulates PD-L1-PD-1 interaction and decreases PD-L1 translocation and stabilization

Because 2-DG suppressed cell-surface PD-L1 expression levels, we next sought to determine whether 2-DG affects PD-L1 stability. Time-lapse images showed that 2-DG significantly reduced the amount of PD-1 protein bound to

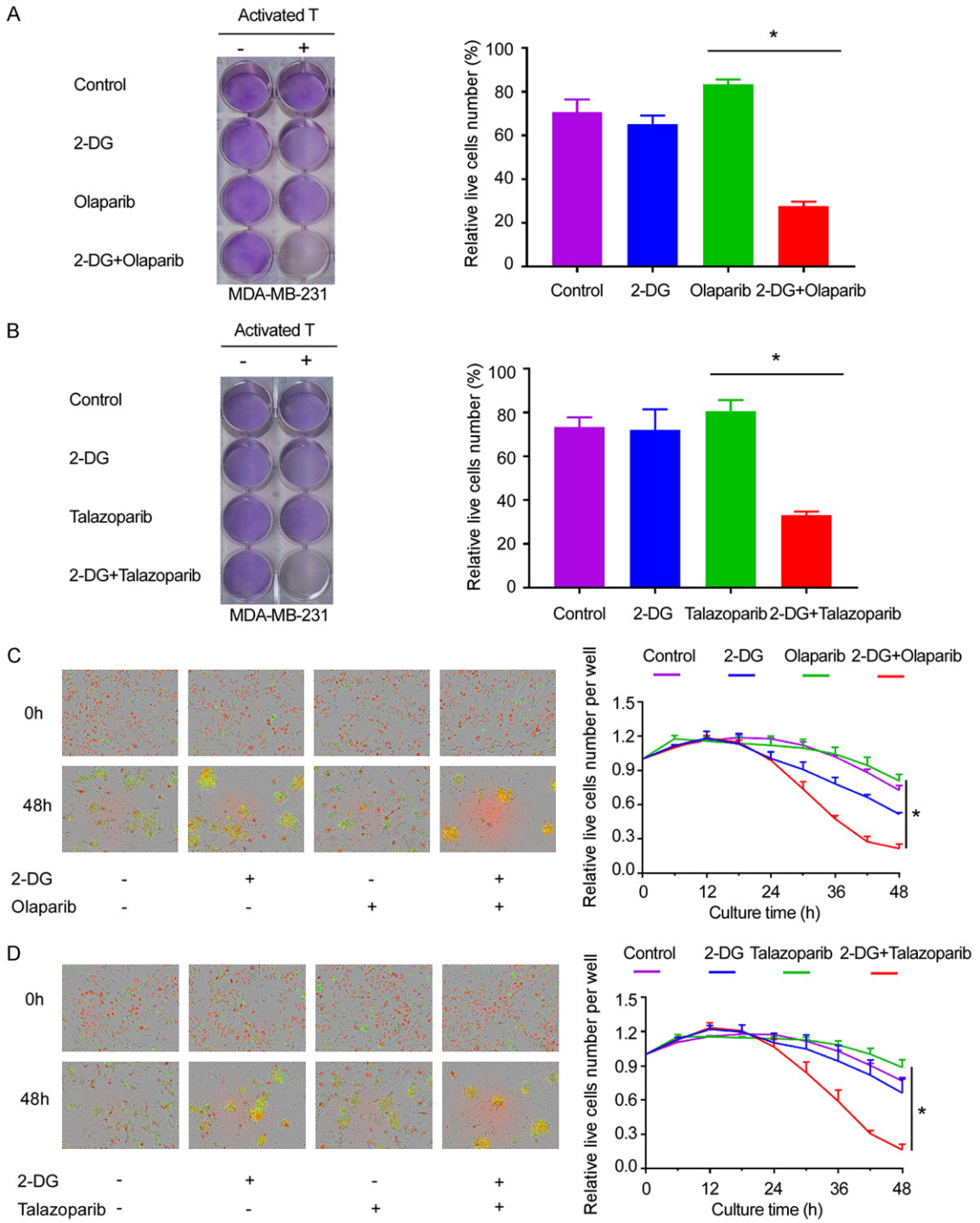

Figure 4. 2-DG enhances T-cell killing efficiency. A. PBMCs were co-cultured with MDA-MB-231 cells at a 15:1 ratio for 48 hours and treated with 2 mmol/L 2-DG and/or 10 μmol/L olaparib. Left, the cells were stained with crystal violet. Right, the percentages of live MDA-MB-231 cells co-cultured with activated PBMCs (normalized to MDA-MB-231 cells cultured without PBMCs) at 48 hours. *P<0.05. B. MDA-MB-231 cells were cultured as those shown as in panel and cells were then treated with 2 mmol/L 2-DG and/or 10 nmol/L talazoparib. Left, the cells were stained with crystal violet. Right, the percentage of live MDA-MB-231 cells co-cultured with activated PBMCs (normalized to those cultured without PBMCs) at 48 hours. *P<0.05. C. MDA-MB-231 cells expressing nuclear red fluorescent protein (RFP) seeded in the 96-well plate were first treated as those shown in panel a. Left, representative merged images showing red fluorescent (nuclear-restricted RFP) and green fluorescent (caspase-3/7 substrate) objects. Images were captured using an IncuCyte Zoom microscope. Right, The number of live cells (red fluorescent

objects) at 6 hours interval were counted and normalized to that at the zero time point. *P<0.05. D. MDA-MB-231 cells expressing nuclear RFP were first treated as those shown in panel b were. Left, Representative merged images showing red fluorescent (nuclear-restricted RFP) and green fluorescent (caspase-3/7 substrate) objects. Images were captured using an IncuCyte ZOOM microscope. Right, the number of live cells (red fluorescent objects) were counted at 6 hours interval were counted and normalized to that at the zero time point. *P<0.05.

cell-surface PD-L1 continuously (**Figure 3A**). 2-DG, as a glucose analog, was incorporated into the PD-L1 glycosylation process (**Figure 3B**). In the presence of the protein synthesis inhibitor cycloheximide, the turnover rate of PD-L1 with the treatment of 2-DG was faster than that in the control group (**Figure 3C**). In addition, 2-DG trapped PD-L1 inside the endoplasmic reticulum (**Figure 3D**). These results suggest that 2-DG reduces cell-surface PD-L1 expression levels by reducing the normal glycosylation of PD-L1.

2-DG re-sensitizes PARPi-treated cancer cells to T-cell killing

To understand the functional significance of the downregulation of PD-L1 by 2-DG, we performed a T-cell-mediated killing assay by co-culturing activated human PBMCs with MDA-MB-231 cells treated with 2-DG and/or PAPRi with olaparib or talazoparib. To find the 2-DG concentration that does not significantly affect cell growth, we tested several 2-DG concentrations with or without PARPi with 10 μM olaparib or 10 nM talazoparib; we used 2 mM 2-DG as our final concentration (Figure S1A-C). PARPi-treated cells were strongly resistant to activated T-cell killing, which is consistent with the findings of our previous study [17]. 2-DG sensitized the PARPi-treated cells to T-cell killing, and the combination treatment group (2-DG+ PARPi) had the best killing efficiency (**Figure 4A-D**). These results indicate that 2-DG re-sensitizes PARPi-treated MDA-MB-231 cells to activated T-cell killing.

Discussion

Although the metabolic effects of 2-DG have been well studied, the role of 2-DG in cancer-associated immunity is still largely unknown. The results of our study demonstrate that 2-DG reduces cell-surface PD-L1 expression primarily through the deglycosylation of PD-L1, which resensitized cancer cells to T-cell-mediated cytotoxicity. These data provide a strong rationale for using 2-DG in combination with PARP inhibitors that upregulate PD-L1 in TNBC.

2-DG is most frequently used to inhibit glucose metabolism. 2-DG is phosphorylated by hexokinase, resulting in the depletion of intracellular ATP and the induction of autophagy [18]. However, 2-DG's high dose requirement as a single agent (65-100 mg/kg body weight) [19, 20] limits its application in the clinic. Several recent studies have reported that 2-DG enhances the anticancer effects of other drugs in prostate, lung, and breast cancer [21-24]. Because glucose is also a key source of polysaccharide compositions on glycoproteins, the energy-independent function of glucose for glycoproteins in tumor progression is of interest. By depleting the cell of available glucose, 2-DG also inhibits protein glycosylation, trapping proteins in the endoplasmic reticulum and triggering the unfolded protein response, which can induce apoptosis [25, 26]. 2-DG is a glucose analog that can be incorporated into cells via glucose transporters. In our study, we found that 2-DG could be incorporated into the glycoprotein (**Figure 3A**), which might change the glycosylation structure and thereby affect various biological functions related to glycoproteins.

Our previous study showed that, although glycosylation is involved in many co-inhibitory signaling interactions, co-stimulatory signaling does not require glycosylation [12]. 2-DG might change the glycan structure of the inhibitory interaction proteins. Thus, 2-DG might have a more substantial effect on relieving the immunosuppressive status of tumors by 1) restricting the energy for cancer cell metabolism, 2) deglycosylating PD-L1 for degradation, and 3) disrupting PD-L1-PD-1 interaction. Therefore, combination 2-DG could re-sensitize the drugs that induce PD-L1 expression, which might play an important role in the resistance.

Previous studies showed that immune checkpoint blockade has promise for the treatment of TNBC. However, antibodies targeting PD-1 or PD-L1 did not elicit a satisfactory response rate [27-29] and offered a survival benefit no better than that achieved with traditional treatment in patients with metastatic TNBC. Only a minority of patients benefit from the therapy, and the

predictive biomarkers for patient selection are not clear. Improving the response rates, the durability of response and overall survival remain challenges in practice. Combination strategies with potential synergistic treatment, primarily with chemotherapy, radiotherapy, targeted therapies, as well as other immune therapies, to improve overall response rate and OS are actively studied in various clinical trials [30-34]. Our previous study showed that combination therapy with gefitinib and an anti-PD-1 antibody could enhance the treatment result [10].

In this study, we found that 2-DG reduced the PD-L1 expression on the cell surface, which induce PD-L1 trapped inside the endoplasmic reticulum. We demonstrated that 2-DG reverses the PARPi-induced upregulation of PD-L1 by deglycosylating PD-L1 in TNBC. Our results provide a strong scientific base for investigating the combination of 2-DG and PARPi in TNBC further.

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Disclosure of conflict of interest

None.

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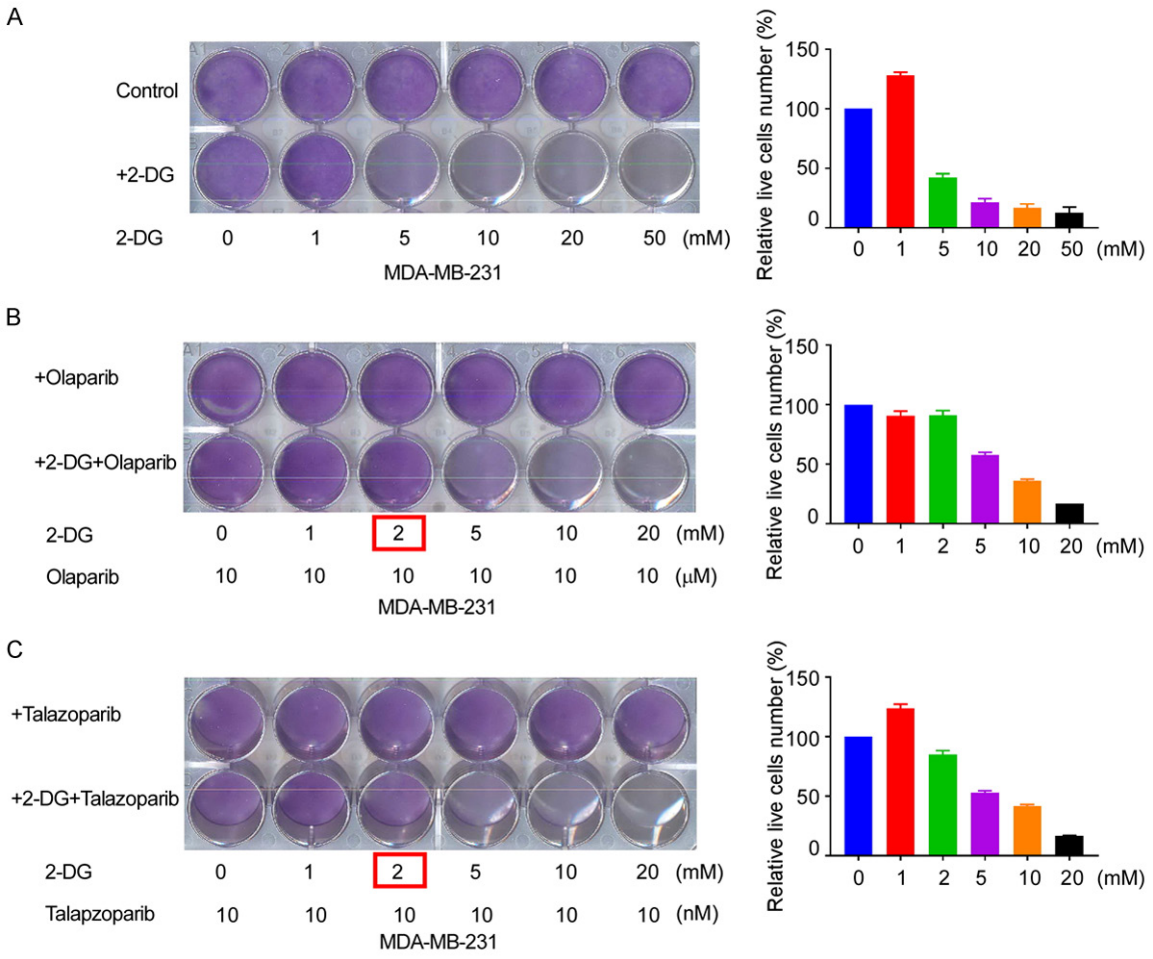

Figure S1. Identifying the 2-DG concentration that does not significantly affect cell growth. A. MDA-MB-231 cells were treated with 1, 5, 10, 20, or 50 mmol/L 2-DG for 48 hours. Left, the cells were stained with crystal violet. Right, the percentage of live cells observed at 48 hours (normalized to the control group). B. MDA-MB-231 cells were treated with 10 μmol/L olaparib and/or 1, 2, 5, or 10 mmol/L 2-DG for 48 hours. Left, the cells were stained with crystal violet. Right, The percentage of live cells at 48 hours (normalized to the control group). C. MDA-MB-231 cells were treated with 10 nmol/L talazoparib and/or 1, 2, 5, or 10 mmol/L 2-DG for 48 hours. Left, cells were stained with crystal violet. Right, The percentage of live cells at 48 hours (normalized to the control group).