Received: 3 January 2020 | Revised: 12 February 2020 | Accepted: 15 February 2020

DOI: 10.1002/mc.23170

RESEARCH ARTICLE

Molecular Carcinogenesis WILEY

Saccharide analog, 2-deoxy-D-glucose enhances 4-1BB-mediated antitumor immunity via PD-L1 deglycosylation

Bareun Kim[1] | Ruoxuan Sun[1] | Wonkyung Oh[1] | Alyssa Min Jung Kim[1] | Johann Richard Schwarz[1] | Seung-Oe Lim[1,2,3]

[1]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana

[2]Purdue Institute of Drug Discovery, Purdue University, West Lafayette, Indiana

[3]Purdue Center for Cancer Research, Purdue University, West Lafayette, Indiana

Correspondence
Seung-Oe Lim, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue Institute of Drug Discovery, Purdue Center for Cancer Research Purdue University, West Lafayette, IN 47907.
Email: limsoe@purdue.edu

Funding information
Purdue Center for Cancer Research; Susan G. Komen, Grant/Award Number: CCR17488088; Purdue Institute for Drug Discovery; Ralph W. and Grace M. Showalter Research Trust Fund

Abstract

Triple-negative breast cancer (TNBC) lacks a well-defined molecular target and is associated with poorer outcomes compared to other breast cancer subtypes. Programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) blockade therapy shows a 10% to 20% response rate in TNBC patients. Our previous studies show that PD-L1 proteins are heavily glycosylated in TNBC, and the glycosylation plays an important role in the PD-L1 protein's stability and immunosuppressive function. However, a strategy for PD-L1 deglycosylation in TNBC is poorly defined. Here we found that a saccharide analog, 2-deoxy-D-glucose (2-DG), inhibits glycosylation of PD-L1 and its immunosuppressive function by combining with EGFR inhibitor, gefitinib. Interestingly, 2-DG/gefitinib-induced deglycosylation of PD-L1 decreased the expression level of PD-L1 protein as well as its binding with PD-1. However, there was no significant decrease in 4-1BB expression and its binding with 4-1BBL by 2-DG/gefitinib. Furthermore, we demonstrated that the combination treatment of 2-DG/gefitinib and 4-1BB antibody enhances antitumor immunity in TNBC syngeneic murine models. Together, our results suggest a new immunotherapeutic strategy to enhance antitumor immunity by PD-L1 deglycosylation and 4-1BB stimulation in TNBC.

KEYWORDS

4-1BB, glycosylation, PD-L1, triple-negative breast cancer

1 | INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for approximately 15% to 20% of breast cancers and lacks the expression of estrogen receptor and progesterone receptor as well as the amplification of human epidermal growth factor receptor 2 (HER2). Due to heterogeneity and lack of well-defined molecular targets of TNBC, treatment of TNBC patients is a challenge, and the patients show a poorer survival compared to other breast cancer subtypes.[1–3] TNBC patients initially respond to conventional chemotherapy, but the disease frequently relapses, leading to patient mortality. Therefore, there is an unmet clinical need for new therapeutic strategies to treat TNBC patients.

Cancer immunotherapy utilizes the patient's immune system to treat cancer. Immune checkpoint blockade, one of the most promising cancer immunotherapies, has demonstrated success in multiple cancer types.[4–6] Given the promising and durable clinical responses, the U.S. Food and Drug Administration approved one cytotoxic T lymphocyte antigen 4 (CTLA-4) antibody, ipilimumab, three programmed cell death protein 1 (PD-1) antibodies, nivolumab, pembrolizumab, and cemiplimab, and three programmed death-ligand 1 (PD-L1/B7-H1) antibodies, atezolizumab, avelumab, and durvalumab for multiple types of cancer.[7–9] PD-1/PD-L1 pathway blockade using PD-1 or PD-L1 antibodies has elicited durable clinical responses in cancer patients by normalizing the imbalanced antitumor

immunity.[10] Despite the improvement in therapeutic efficacy, the response rate remains modest at 10% to 40% in the clinic. In TNBC, due to immunogenicity and high expression of PD-L1, PD-1/PD-L1 blockade therapy is emerging as a potential therapy.[11] However, the PD-1/PD-L1 blockade therapy using PD-1 or PD-L1 antibodies in TNBC was not satisfactory because the PD-L1 antibody, atezolizumab, showed a 10% to 20% response rate in TNBC patients.[12,13] Therefore, new immunotherapeutic strategies to treat TNBC patients are urgently needed.

To improve the current PD-1/PD-L1 therapeutic efficacy and prediction of response rate, further understanding of the regulatory mechanism of the PD-1/PD-L1 pathway in tumor microenvironments is necessary. PD-L1, a 33 kDa type 1 transmembrane protein that binds to PD-1, is a key protein in the PD-1/PD-L1 blockade pathway. The engagement of PD-L1 expressing tumor cells and PD-1 expressing T cells suppresses antitumor immunity by T cell dysfunction in the tumor microenvironment.[10] The function of PD-L1 protein and its stability are regulated by posttranslational modification (PTM) such as phosphorylation, ubiquitination, and glycosylation.[8,14–17] Recently, the regulation of PTMs on PD-L1 protein are being intensively studied. Aberrant alteration of PTMs modulates PD-L1-mediated immune responses. Several preclinical studies show that targeting the PTMs of PD-L1 is one of the promising therapeutic strategies in multiple cancer types including TNBC.[8,14–17] Among several types of PTM in PD-L1 protein, the glycosylation of PD-L1 is required for the PD-1/PD-L1 interaction and its immunosuppressive function.[14,17] In our previous studies, PD-L1 is heavily glycosylated in TNBC,[14] and targeting the glycosylated PD-L1 specific antibody eradicates TNBC cells.[17] These findings support that targeting glycosylation of PD-L1 is one of the most effective strategies to improve the therapeutic efficacy of current PD-1/PD-L1 blockade therapies.

In addition to PD-1/PD-L1 blockade, the manipulation of the tumor necrosis factor (TNF) family signaling pathway by agonist antibodies can directly stimulate the immune cells in the immunosuppressive tumor microenvironment.[10] For instance, GITR, OX-40, and 4-1BB (CD137) agonist antibodies are currently being studied in clinical trials and have shown promising results as a single agent or in combination with PD-1/PD-L1 blockade therapy for several cancer types.[18–20] Among the TNF receptor superfamily, the 4-1BB expression is restricted to primed $CD4^+$ and $CD8^+$ T cells.[21] 4-1BB signaling by engaging with 4-1BB ligand (4-1BBL) or agonist antibody stimulates activity and growth of $CD8^+$ T cells and NK cells preferentially.[22,23] Although the therapeutic efficacy of a single 4-1BB antibody (urelumab or utomilumab) is not satisfactory due to liver toxicity or low efficacy in clinical trials,[24] the combination of 4-1BB antibody and PD-1/PD-L1 blockade has shown promising therapeutic efficacy in advanced solid tumors (NCT02554812). Thus, the scientific rationales support the combination of agonist antibodies of the TNF receptor superfamily and PD-1/PD-L1 blockade therapy. However, further studies are required to optimize a combination treatment strategy and improve the therapeutic efficacy in the clinic.

In this study, we explored a new immunotherapeutic strategy to treat TNBC patients by combining inhibition of PD-L1 glycosylation with TNF receptor family agonist antibody-induced T cell activation. Here, we identified the effective combination of saccharide analog, 2-deoxy-D-glucose (2-DG) and EGFR inhibitor, gefitinib, which inhibits PD-L1's glycosylation and expression but not 4-1BB expression and its function. We further evaluate the therapeutic efficacy of our new combination treatment of 2-DG/gefitinib and 4-1BB antibody in TNBC syngeneic murine models.

2 | METHODS

2.1 | Cell culture, transfection, and treatment

Human breast cancer cell line BT-549 and MDA-MB-231, human epidermoid cancer cell line A431, and mouse breast cancer cell lines 4T1 and EMT6 were obtained from ATCC (Manassas, VA). Human embryonic kidney cell line HEK293FT was obtained from Thermo Fisher Scientific (Waltham, MA). Cells were grown in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with 10% fetal bovine serum. EGF (BioLegend, San Diego, CA) was prepared according to the manufacturer's instructions. For stable expression of stimulatory immune receptors, complementary DNA of human 4-1BB, OX-40, and GITR (Sino biological, Wayne, PA) was inserted between the *Nhe*I and *Not*I site of pCDH-CMV vector (System Biosciences, Palo Alto, CA). Lentivirus was packaged by co-transfecting transfer plasmids with pMD2.G (#12259; Addgene) and psPAX2 (#12260; Addgene) at a ratio of 4:2:3 to HEK293FT cells with X-tremeGENE HP (Roche Diagnostics, Indianapolis, IN), and the supernatant was harvested for lentiviral transduction. Selection with 2 μg/mL puromycin was routinely performed to maintain ectopic gene expression. All constructs were verified by Sanger sequencing. PD-L1 overexpressed BT-549 cell lines with endogenous PD-L1 knockdown was established using pGIPZ-shPD-L1/Flag-PD-L1 vector as described.[14,17] Cells were treated with 50 ng/mL EGF, and 5 μM gefitinib was used to inhibit EGFR kinase activity. All saccharide analogs were used for inhibition of PD-L1 glycosylation.

2.2 | Animal procedure

All BALB/c mice (6-8-week-old females; Jackson Laboratories, Bar Harbor, ME) procedures were conducted under the guidelines approved by the Purdue Animal Care and Use Committee (PACUC) at Purdue University. 4T1 or EMT6 cells (1×10^5 cells in 25 μL of medium mixed with 25 μL of Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA) were injected into the mammary fat pad of 6-week-old female BALB/c mice (Jackson Laboratories). Mice were divided according to the mean value of tumor volume in each group. For drug treatment, mice were treated with daily oral doses 500 mg/kg 2-DG (Alfa Aesar, Haverhill, MA) and 10 mg/kg gefitinib (LC Laboratories, Woburn, MA) for 2 weeks (5 days/week). 4-1BB antibody (LOB12.3, Bio X Cell, West Lebanon, NH) or control mouse IgG (Bio X Cell) were administered at 2.5 mg/kg intraperitoneally on days 4, 7, 10, and 13 after tumor cell inoculation (n = 8 mice per

group). Tumor was measured with a caliper, and tumor volume was calculated by the formula: $\pi/6 \times \text{length} \times \text{width}$,[2] where length is the longest diameter and width is the shortest diameter. Statistical analysis was carried out using a paired Student's *t* test and assumed to be significant at * indicates $P < .05$. ns means not significant.

2.3 | Immunofluorescence for mouse tumor tissues

Tumor masses were frozen in an OCT block immediately after excision. Cryostat sections of 5-μm thickness were attached to saline-coated slides. Cryostat sections were fixed with 4% paraformaldehyde for 30 minutes at room temperature and blocked with blocking solution (1% bovine serum albumin, 2% donkey and/or chicken serum, and 0.1M PBS) at room temperature for 30 minutes. Samples were stained with primary antibodies against PD-L1, CD8, and granzyme B overnight at 4°C, followed by secondary antibodies at room temperature for 1 hour. Nuclear staining was performed with Hoechst 33342 (Thermo Fisher Scientific). The stained sections were visualized by automated microscopy (Lionheart LX; BioTek Instruments, Inc, Winooski, VT). Granzyme B and PD-L1 positive area and the number of CD8 positive CTL were assessed per high power field (200×). Fourteen randomly chosen microscope fields from four serial sections in each tissue block were examined for the number of CD8 positive CTL and granzyme B or PD-L1 positive areas for each tissue.

2.4 | Tumor infiltration lymphocyte profile analysis

Excised tumors underwent a mechanical and enzymatic dissociation using an automatic cell dissociator (gentleMACS Dissociator; Miltenyi Biotec, Bergisch Gladbach, Germany) after being sectioned into pieces of approximately 1-2 mm^3. The isolation procedure for tumor infiltrating lymphocytes followed the manufacturer's instructions in the Tumor Dissociation Kit (130-096-730; Miltenyi Biotec). After Percoll (GE) gradient centrifugation, immune cells were collected from the interface between 40% and 80% Percoll. T cells were stained with CD3ε-PerCP (145-2C11; BioLegend), CD8a-BV605 (53-6.7; BioLegend), IFN-γ-BV421 (XMG1.2; BioLegend), CD45-BV421 (30-F11; BioLegend), CD11b-PerCP (M1/70; BioLegend), and Gr-1-PE (RB6-8C5; BioLegend). Stained samples were analyzed using the BD FACS Fortessa LSR (BD Bioscience) flow cytometer.

2.5 | In vitro immune receptor and ligand interaction assay

To measure immune receptor and ligand interaction, His-tagged proteins were incubated with or without Rapid PNGase F (New England BioLabs, Ipswich, MA) in nonreducing buffer for 30 minutes at 50°C and then placed on a nickel-coated 96-well plate. The plate was then incubated with recombinant Fc-tagged protein for 1 hour. The secondary antibodies used were anti-human IgG Fc-specific Alexa 488 dye conjugate (Jackson ImmunoResearch Inc, West Grove, PA). Fluorescence intensity of Alexa flour 488 dye was measured by a microplate reader (Synergy Neo2; BioTek Instruments, Inc).

2.6 | Mice immunization

Six-week-old BALB/c mice were purchased from Jackson Laboratories. Mice were injected subcutaneously with 100 μg of $OVA_{323-339}$ (Invivogen) mixed with adjuvant or phosphate buffered saline (PBS) per mouse three times at 2-week intervals. One week after the final injection, mononuclear cells from the spleens and lymph nodes were harvested for preparation of OVA-specific T-cell lines and clones.

2.7 | OVA-specific T-cell clones and lines

One-week after the third immunization with $OVA_{323-339}$, the draining of lymph nodes and spleens of BALB/c mice were collected and used to prepare a single-cell suspension (2×10^6 cells/mL) in R10 media. $OVA_{323-339}$ pulsed syngeneic spleen and lymph node cells were stimulated with 100 μg/mL $OVA_{323-339}$ and expanded and activated with 2 μg/mL CD28 antibody and 10 ng/mL mouse IL-2 coated with 10 μg/mL CD3ε antibody in a six-well plate for 5 days at 37°C in 5% CO_2.

2.8 | T cell-mediated tumor cell killing assay

The T cell-mediated tumor cell killing assay was performed according to the previous description.[14] To analyze the killing of tumor cells by T-cell inactivation, a nuclear-restricted red fluorescent protein (RFP) and cytosolic ovalbumin expressing 4T1 cells were cocultured with $OVA_{323-339}$ specific T cells. $OVA_{323-339}$ specific T cells were activated by incubation with 100 ng/mL CD3ε antibody (Biolegend) and mouse 10 ng/mL IL-2 (Biolegend). After 96 hours, RFP signals were measured as survived tumor cells.

2.9 | Western blot analysis

Cells were harvested after PBS washes in lysis buffer (1.25M urea and 2.5% sodium dodecyl sulfate) followed by sonication and centrifugation. Protein concentration was measured by the bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford, IL). Immunoblotting was performed with primary antibodies against human PD-L1 (1:1000; 13684; Cell Signaling Technology, Danvers, MA), mouse PD-L1 (1:1000; EPR20529; Abcam, San Francisco, CA), 4-1BB (1:1000, 18798; Cell Signaling Technology), GITR (1:1000; 10419; Cell Signaling Technology), OX-40 (1:1000; 15123; Cell Signaling Technology), Flag (1:1000; 14793S; Cell Signaling Technology), His (1:1000; 2366S; Cell Signaling Technology), and β-actin (1:5000; MA5-15739; Invitrogen). Image acquisition and quantification of band intensity were performed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.10 | Quantification and statistical analysis

Data in bar graphs represent mean ± standard deviation (SD) fold change relative to untreated or control groups for three independent experiments. Student's *t* test was performed for experimental data. For multivariate data analysis, a one-way or two-way analysis of variance, followed by Scheffe's post hoc test, was used for assessment of group differences. A $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | The combination of saccharide analog and EGFR inhibitor inhibits glycosylation of PD-L1 in TNBC

Glycosylation of PD-L1 protein plays an important role in its stability and immunosuppressive function.[14,17] Thus, inhibition of glycosylation could serve as a potential therapeutic strategy for TNBCs. To investigate which saccharide analog inhibits glycosylation of PD-L1, BT-549 cells were treated with six different saccharide analogs. We also included the N-linked glycosylation inhibitor, tunicamycin (TM), treated cells as a control for nonglycosylated PD-L1 protein. Among the panel of saccharide analogs we tested, 2-DG significantly inhibited PD-L1 glycosylation as well as protein expression of PD-L1 in BT-549 and other cell lines (Figure 1A and S1A, S1B). As shown in Figure 1B, the glycosylation level of PD-L1 was reduced in a dose-dependent manner. Since 2-DG suppresses glucose metabolism by blocking hexokinase activity, we sought to examine whether 2-DG-induced inhibition of PD-L1 glycosylation is associated with glycolysis inhibition. Interestingly, 3-bromopyruvate (3-BP), another hexokinase inhibitor, did not inhibit PD-L1 glycosylation, suggesting that 2-DG induces deglycosylation of PD-L1 protein through a glycolysis-independent mechanism (Figure 1C). In our previous study, EGF signaling stabilized PD-L1 protein by enhancing its glycosylation in TNBC cells.[14,17] This prompted us to examine the effect of combination

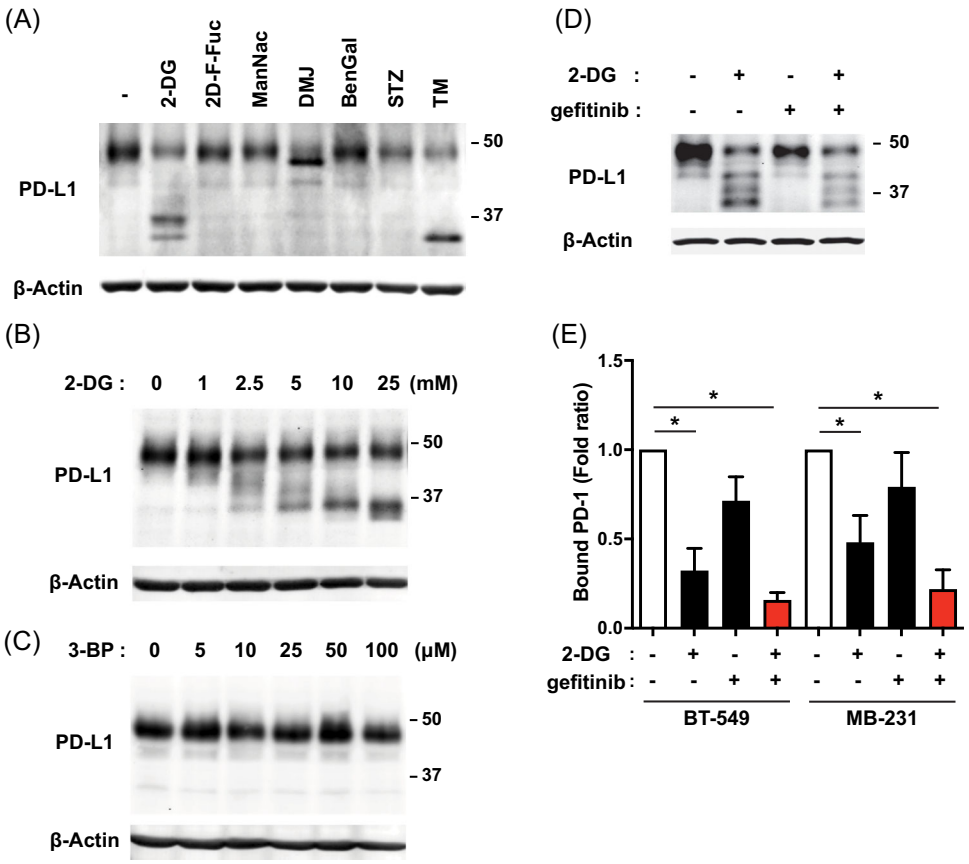

FIGURE 1 The combination of saccharide analog and EGFR inhibitor inhibits glycosylation of PD-L1 in TNBC. A, PD-L1 expression detected by the PD-L1 antibody in BT-549 cells. BT-549 cells were treated with 10 mM 2-DG, 10 mM 2-D-F-Fuc, 10 mM ManNac, 10 mM DMJ, 5 mM BenGal, 10 mM STZ, or 2.5 μg/mL tunicamycin (TM) overnight. B, BT-549 cells were treated with 0, 1, 2.5, 5, 10, and 25 mM of 2-DG for 16 hours, and then PD-L1 expression was analyzed by western blot analysis. C, BT-549 cells were treated with 0, 5, 10, 25, 50, and 100 μM of 3-BP for 16 hours and then PD-L1 expression was analyzed by western blot analysis. D, BT-549 cells were treated with 10 mM 2-DG, and/or 5 μM gefitinib. PD-L1 expression was analyzed by western blot analysis. β-Actin served as a loading control. E, PD-1 binding assay in BT-549 and MDA-MB-231 cells treated with 2-DG and/or gefitinib. β-Actin served as a loading control. *Statistically significant ($P < .05$). All error bars indicate the mean ± standard deviation of three independent experiments. PD-L1, programmed death-ligand 1; TNBC, triple-negative breast cancer [Color figure can be viewed at wileyonlinelibrary.com]

treatment of EGFR tyrosine kinase inhibitor (TKI), gefitinib, and 2-DG on PD-L1's glycosylation. Indeed, the combination treatment of 2-DG and gefitinib inhibited PD-L1 glycosylation and the PD-1 protein binding on a membrane in TNBC cells (Figure 1D,E, and S1C). These results imply that the combination treatment of 2-DG and gefitinib can enhance antitumor immunity by deglycosylation of PD-L1 in TNBC.

3.2 | The glycosylation is not required for the interaction of the costimulatory receptor, 4-1BB

To determine whether glycosylation is required for ligand and receptor interaction, we performed an in vitro receptor-ligand binding assay to assess the interaction between Fc-tagged receptor and His-tagged glycosylated or nonglycosylated ligands. We examined the interactions of five coinhibitory receptor/ligand pairs and five costimulatory receptor/ligand pairs. To remove glycosylation of immune receptors/ligands, we treated His-tagged proteins with PNGase F. Bands that corresponded to higher-molecular-weight PD-L1, PD-L2, PD-1, CTLA-4, TIM-3, 4-1BB, OX-40, GITR, CD40, and ICOSL (Figure 2A; black circle, glycosylated proteins; red circle, unglycosylated proteins) were reduced upon PNGase F treatment. All five coinhibitory receptor/ligand pairs showed significant loss in binding upon PNGase F treatment (Figure 2B). However, the nonglycosylated form of all five costimulatory receptors/ligands did not lose the binding affinity with their ligands (Figure 2B). These results suggest that glycosylation is required for the interaction of co-inhibitory receptors/ligands, but not costimulatory receptors/ligands.

In addition, to explore whether combination treatment of 2-DG and gefitinib affects protein expression of immune receptors, we analyzed the expression level of immune receptors on T cells isolated from gefitinib and/or 2-DG treated 4T1 tumors in BALB/c mice. There was no significant alteration of coinhibitory or costimulatory receptors' expression on T cells (Figure 2C), although the protein expression of PD-L1 on tumor cells was reduced upon gefitinib and 2-DG treatment (Figure 1D). Next, among three costimulatory receptor agonist antibodies, we observed that 4-1BB and GITR antibodies promoted OVA-specific T cell-killing activity in 4T1-OVA cells (Figure 2D,E). We also found that 2-DG/gefitinib treatment did not significantly decrease the expression level of 4-1BB protein (Figure 2F). However, the expression level of OX-40 and GITR proteins were reduced by 2-DG/gefitinib treatment (Figure 2F). These data imply that 2-DG/gefitinib inhibits PD-L1′s function preferentially on tumor cells, but has no significant effect on the costimulatory receptor, 4-1BB, on T cells through deglycosylation of PD-L1. These results also suggest that PD-L1 deglycosylation by 2-DG/gefitinib may not reduce the therapeutic efficacy 4-1BB agonist antibody.

3.3 | The combination treatment of PD-L1 deglycosylation and 4-1BB stimulation synergistically enhances antitumor immunity in TNBC

We hypothesized that the combination treatment of 4-1BB antibody and PD-L1 deglycosylation by 2-DG/gefitinib synergistically enhances antitumor immunity in TNBC because PD-L1 deglycosylation by 2-DG/gefitinib may not reduce the therapeutic efficacy of 4-1BB agonist antibody. To this end, we treated 4T1 or EMT6 tumors with 2-DG/gefitinib (S/T) and/or 4-1BB antibody in BALB/c mice (Figure 3A). The 4T1 and EMT6 tumor sizes were significantly decreased in 2-DG/gefitinib (S/T) and 4-1BB antibody-treated mice. No significant changes in body weight and minimal cytotoxicity in the liver and kidney were observed (Figures 3B-E and S3). To validate the therapeutic efficacy of the combination treatment on antitumor immunity, we analyzed TIL in 2-DG/gefitinib and/or 4-1BB antibody-treated tumors. Tumors resected from each mouse were subjected to immunofluorescence (IF) staining and flow cytometric analysis. The combination treatment of 2-DG/gefitinib (S/T) and 4-1BB antibody reduced the PD-L1 level and increased the cytotoxic T cell activation indicator, granzyme B, and activated the $CD8^+$ cytotoxic T cell ($CD3^+CD8^+IFN\gamma^+$) population (Figure 4A-E). We also assessed the myeloid-derived suppressor cell (MDSC; $CD45^+CD11b^+Gr\text{-}1^+$) population because MDSCs suppress both innate and adaptive immunity within the tumor microenvironment.[25,26] MDSCs population were decreased in 2-DG/gefitinib and/or 4-1BB antibody treated tumors (Figure 4F). Together, the combination treatment of 2-DG/gefitinib and 4-1BB agonist antibody synergistically enhances antitumor immunity in TNBC murine models.

4 | DISCUSSION

We demonstrated that our new combination treatment of 2-DG/gefitinib and 4-1BB antibody enhances antitumor immunity in TNBC syngeneic murine models. In the current study, we explored a new immunotherapeutic strategy to treat TNBC patients by PD-L1 deglycosylation. Our previous study showed that PD-L1 protein is highly glycosylated in TNBC, and glycosylation of PD-L1 suppresses antitumor immunity via stabilization of PD-L1 protein.[14] N-linked glycosylation (glycosylation) plays a critical role in determining protein structure and function. In particular, glycosylation of membrane receptor proteins is important for protein-protein interaction.[27] Glycoprotein has a unique and complex glycan structure, and it is composited with a saccharide. It implies that a saccharide analog can play a role as an inhibitor of glycosylation. Thus, we sought a new strategy for PD-L1 deglycosylation through saccharide analogs to enhance antitumor immunity in TNBC.

Here, we identified the effective combination of 2-DG and gefitinib for PD-L1 deglycosylation in TNBC. 2-DG is known as a glycolysis inhibitor (or hexokinase inhibitor), and its effects on the metabolism have been well studied.[28] However, the role of 2-DG

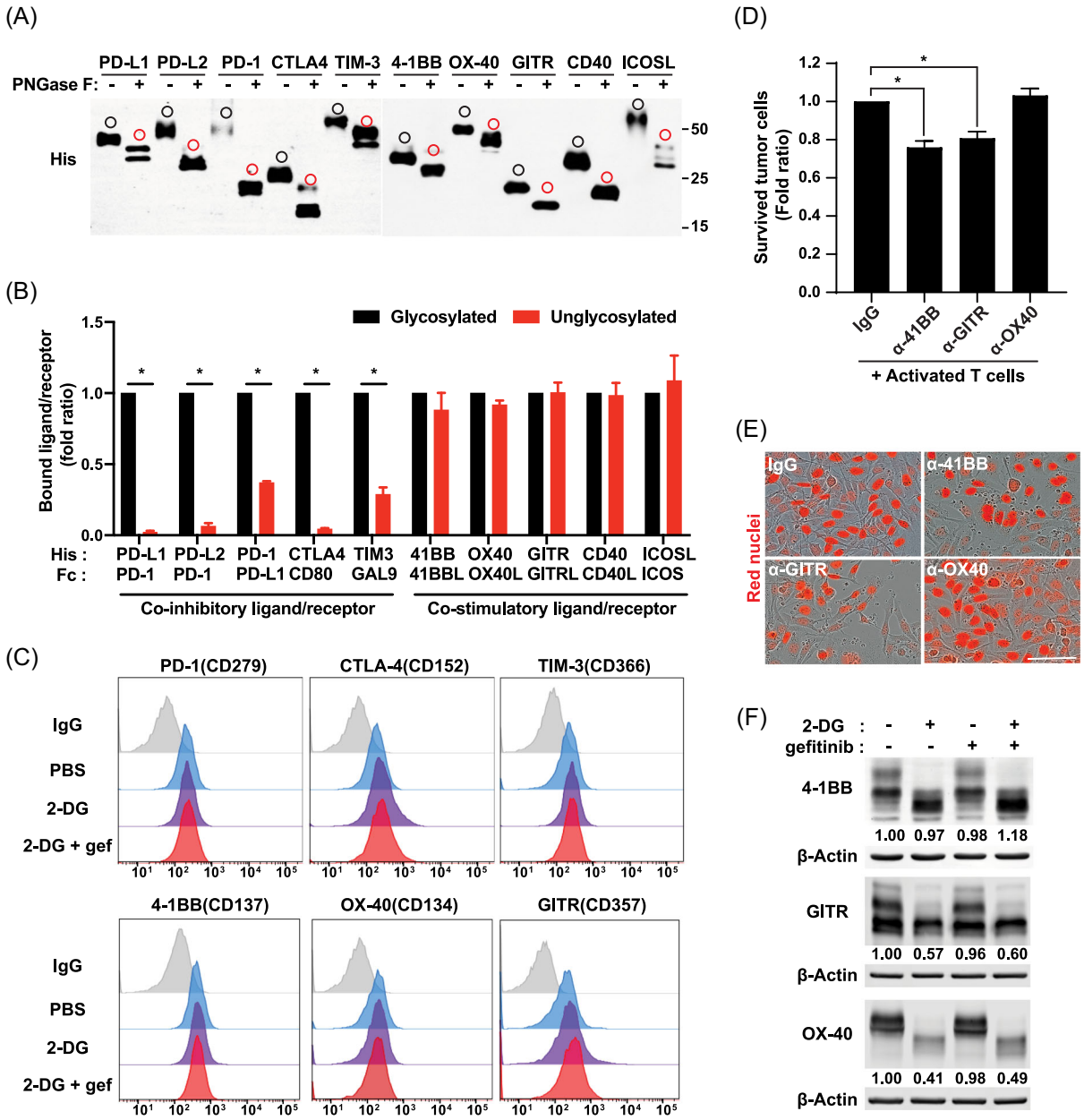

FIGURE 2 The glycosylation is not required for the interaction of the costimulatory receptor, 4-1BB. A, Western blot analysis of His-tagged receptor/ligand proteins. Protein samples were pretreated with or without PNGase F for 30 minutes before western blot analysis. black circle, glycosylated proteins; red circle, unglycosylated proteins. B, In vitro association of immune ligand/receptor pairs. Ratio of 1.0 indicates no change of binding upon PNGase F treatment. C, Flow cytometry measuring coinhibitory and stimulatory receptors' expression on the membrane of $CD3^+$ tumor infiltrating lymphocytes (TILs). TILs were isolated from 2-DG and/or gefitinib treated 4T1 tumors in BALB/c mice. D,E, T cell-mediated tumor cell killing assay in 4T1-OVA cells. T cells were isolated from $OVA_{323-339}$ pulsed BALB/c mice and then were activated with CD3 antibody (100 ng/mL) and IL-2 (10 ng/mL). 4T1-OVA cells were cocultured with the activated T cells upon 4-1BB, GITR, or OX-40 agonist antibody. The quantitative ratio of live cells is shown in the bar graph (D). Representative phase and red fluorescent (nuclear restricted RFP) merged images (20×) of activated T cell cocultures at 96 hours are shown (E). F, Exogenous 4-1BB, GITR, or OX-40 expression determined by western blot analysis in 2-DG and/or gefitinib-treated 4-1BB, GITR, or OX-40 expressing 293FT cells. *Statistically significant ($P < .05$). All error bars indicate the mean ± standard deviation of three independent experiments. RFP, red flourescent protein [Color figure can be viewed at wileyonlinelibrary.com]

in antitumor immunity remains unclear. Recently, we showed that a glucose analog, 2-DG, can be incorporated into the glycan of the PD-L1 protein.[29] Although 2-DG is one of the most effective drugs for inhibition of glycolysis, 2-DG alone is not effective in cancer cell killing and has a toxicity issue at high doses.[28,30] In our previous study, gefitinib sensitized TNBC cells to relatively low concentrations of 2-DG compared with 2-DG alone.[31] These findings urge us to seek an effective combination of saccharide analog and EGFR inhibitor to inhibit PD-L1's glycosylation and expression.

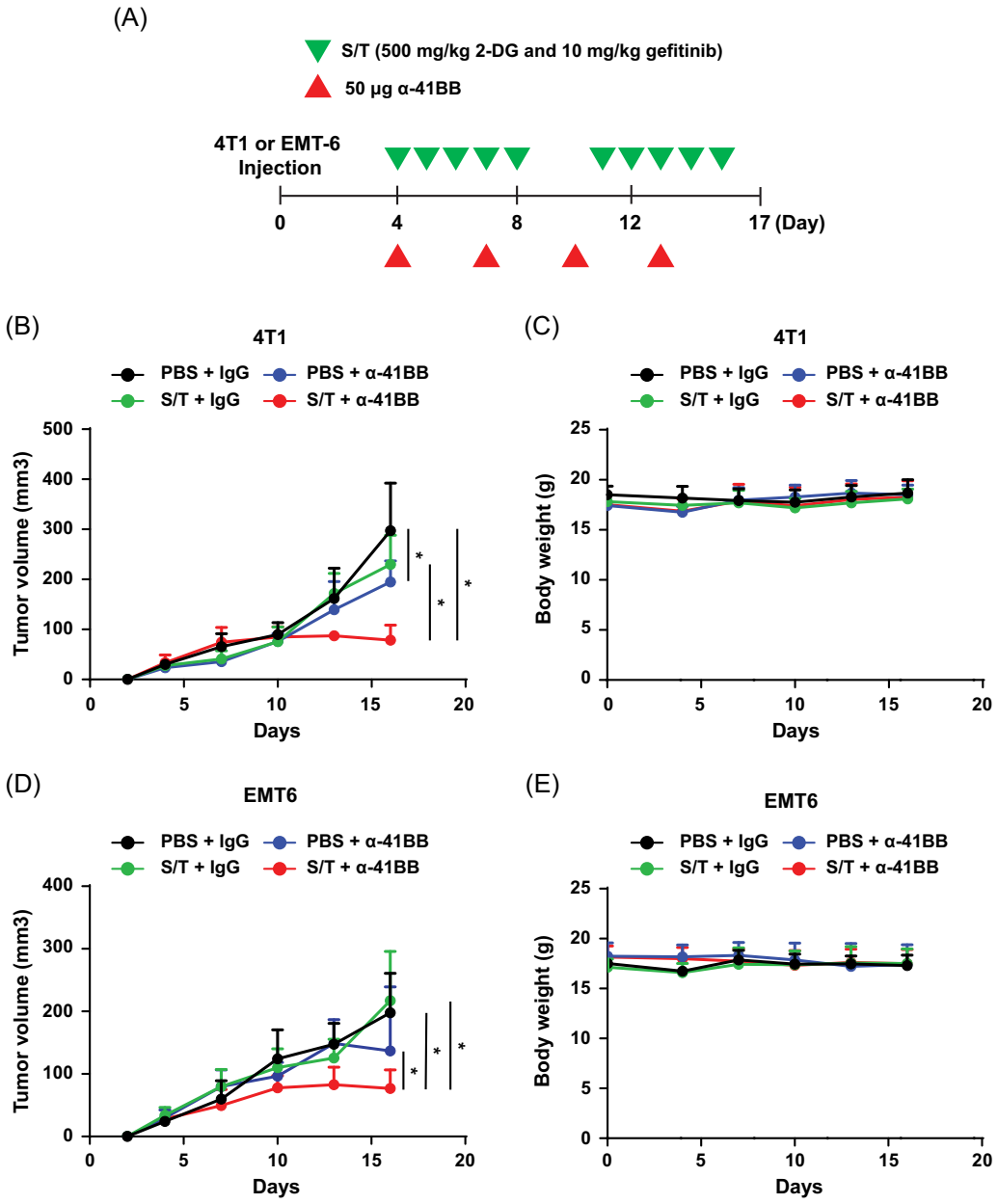

FIGURE 3 The combination treatment of PD-L1 deglycosylation and 4-1BB stimulation synergistically suppresses 4T1 or EMT6 tumor growth in BALB/c mice. A, The treatment protocol is summarized. 4T1 or EMT6 cells were injected into BALB/c mice on day 0. 2-DG/gefitinib (S/T) and 4-1BB agonist antibody (α-4-1BB) were administered as indicated. B, Tumor growth of 4T1 cells in immunocompetent BALB/c mice treated with S/T and/or α-4-1BB. Tumor growth was measured at the indicated time points and dissected at the endpoint (n = 8 mice per group). C, The body weight of S/T and/or α-4-1BB treated mice was measured at the indicated time points (n = 8 mice per group). D, Tumor growth of EMT6 cells in immunocompetent BALB/c mice treated with S/T and/or α-4-1BB. Tumor growth was measured at the indicated time points and dissected at the endpoint (n = 8 mice per group). E, The body weight of S/T and/or α-4-1BB treated mice was measured at the indicated time points (n = 8 mice per group). S/T, 2-DG/gefitinib; *Statistically significant ($P < .05$). All error bars indicate the mean ± standard deviation. PD-L1, programmed death-ligand 1 [Color figure can be viewed at wileyonlinelibrary.com]

Despite the recent success of immune checkpoint blockade therapy such as PD-1 and PD-L1 blockade antibodies, the response rate in cancer patients is 15% to 30%.[6] Thus, there is a clinical need for new immunotherapeutic strategies to treat cancer patients. In this study, we demonstrate that the deglycosylation of PD-L1 by 2-DG/gefitinib is an alternative strategy for PD-L1 blockade. The glycosylation of PD-L1 is required for the PD-1/PD-L1 interaction and its immunosuppressive function.[14,17] PD-L1 is heavily glycosylated in TNBC,[14] and targeting the glycosylated PD-L1 specific antibody eradicates TNBC cells.[17] The removal of N-linked glycosylation of PD-L1 significantly improves the anti-PD-L1 antibody binding affinity.[32] These findings support that the deglycosylation of PD-L1 is

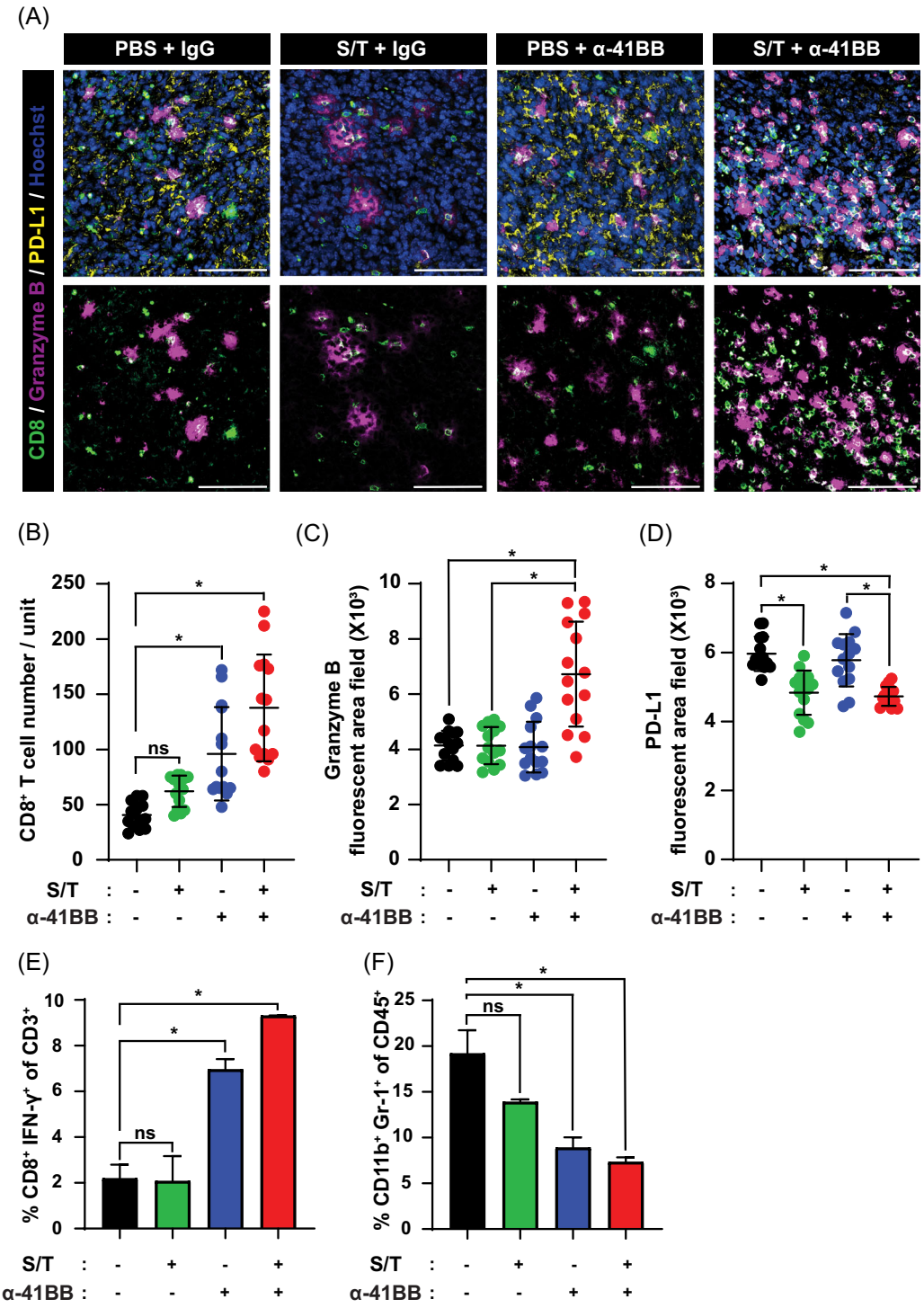

FIGURE 4 The combination treatment of PD-L1 deglycosylation and 4-1BB stimulation synergistically enhanced antitumor immunity in 4T1 syngeneic mouse model. A-D, Immunofluorescence staining of protein expression of PD-L1, CD8, and granzyme B in 4T1 tumor masses from 2-DG/gefitinib (S/T) and/or 4-1BB agonist antibody (α-4-1BB)-treated BALB/c mice. Hoechst, nuclear counterstaining. Scale bar, 100 μm. Representative images of immunostaining of CD8, granzyme B, and PD-L1 in the 4T1 tumor mass (A). CD8 (B), granzyme B (C), and PD-L1 (D) were quantified using Gen5 software (BioTek). n = 14; Four tissue slides per tumor; Four mice per group. Unit = 119 691 μm^2. E, Intracellular cytokine staining of $CD8^+$ $IFN\gamma^+$ cells in $CD3^+$ T cell populations from isolated tumor-infiltrating lymphocytes. n = 8 per group. F, MDSC ($CD45^+CD11b^+Gr\text{-}1^+$) population was analyzed by flow cytometry. n = 8 per group. S/T, 2-DG/gefitinib; *Statistically significant ($P < .05$). ns means not significant. All error bars indicate the mean ± standard deviation. MDSC, myeloid-derived suppressor cell; PD-L1, programmed death-ligand 1 [Color figure can be viewed at wileyonlinelibrary.com]

one of the most effective strategies to improve the therapeutic efficacy of current PD-1/PD-L1 blockade therapies.

We also proposed a new combination treatment of 2-DG/gefitinib and 4-1BB antibody to enhance antitumor immunity in TNBC. The combination of 4-1BB agonist antibody and PD-1/PD-L1 blockade antibodies has shown promising therapeutic efficacy in advanced solid tumors (NCT02554812), although the outcome of a single 4-1BB antibody treatment is not satisfactory due to low efficacy in clinical trials.[24] Thus, our new combination of deglycosylation and 4-1BB antibody to enhance antitumor immunity in TNBC may serve as an alternative strategy to the combination treatment of PD-L1 and 4-1BB antibodies. Although 2-DG/gefitinib inhibited PD-L1 expression and glycosylation, the therapeutic efficacy of 2-DG/gefitinib treatment was not satisfactory in 4T1 and EMT6 syngeneic murine models (Figure 3). To improve the efficacy of 2-DG/gefitinib treatment, we sought to seek a way to enhance T cells immunity using TNF receptor superfamily agonist antibodies. Interestingly, 2-DG/gefitinib did not decrease the expression level of 4-1BB protein expression, and it was demonstrated that glycosylation is not required for 4-1BB/4-1BBL interaction. Furthermore, combining the 4-1BB antibody with PD-1 antibody has demonstrated synergy and diminished toxicities compared with single PD-1 or 4-1BB antibody treatment.[33,34] Thus, the scientific rationales support the combination of 4-1BB agonist antibody and PD-L1 blockade therapy. Indeed, combining 2-DG/gefitinib with a 4-1BB agonist antibody showed substantial synergy in TNBC murine tumor models.

Interestingly, glycosylation is required for the interaction of coinhibitory receptors/ligands, but not costimulatory receptors/ligands (Figure 2B). Specific inhibition of targeted immune receptors by saccharide analogs or glycosylation inhibitors is not available because saccharide analogs or glycosylation inhibitors may inhibit the overall glycosylation process or a specific step of glycosylation. However, our findings imply that deglycosylation of immune receptors by 2-DG or other glycosylation inhibitors may not inhibit the interaction of costimulatory receptors and ligands. It also suggests that saccharide analogs or glycosylation inhibitors may preferentially inhibit coinhibitory immune receptor signaling, but not costimulatory immune receptor signaling.

In this study, we found that 2-DG/gefitinib inhibited PD-L1 glycosylation in TNBC cells. We also demonstrated that our new combination treatment of 2-DG/gefitinib and 4-1BB antibody enhances antitumor immunity in TNBC syngeneic murine models. Our study suggests a new strategy to combat TNBC and also provides a solid scientific base for further study of the combination treatment of PD-L1 deglycosylation and 4-1BB stimulation in other cancer types.

ACKNOWLEDGMENTS

This study was funded in part by Susan G. Komen Breast Cancer Foundation (CCR17488088); Ralph W. and Grace M. Showalter Research Trust grant; Purdue Institute for Drug Discovery (PIDD) Programmatic Area grant; Purdue Center for Cancer Research. The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute. This work was supported by NIH Shared Instrumentation Grant S10DO20029.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

BK and SOL designed the experiments and interpreted the results. BK, RS, WO, AMJK, and JRS conducted the experiments; BK, AMJK, and SOL wrote the manuscript. SOL supervised the entire project.

DATA AVAILABILITY STATEMENT

All the data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Seung-Oe Lim http://orcid.org/0000-0002-8138-4549

REFERENCES

1. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121(7):2750-2767.
2. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol*. 2008;26(15):2568-2581.
3. Metzger-Filho O, Tutt A, de Azambuja E, et al. Dissecting the heterogeneity of triple-negative breast cancer. *J Clin Oncol*. 2012;30(15):1879-1887.
4. Baumeister SH, Freeman GJ, Dranoff G, Sharpe AH. Coinhibitory pathways in immunotherapy for cancer. *Annu Rev Immunol*. 2016;34:539-573.
5. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol*. 2015;33(17):1974-1982.
6. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. 2015;27(4):450-461.
7. Atkins MB, Larkin J. Immunotherapy combined or sequenced with targeted therapy in the treatment of solid tumors: current perspectives. *J Natl Cancer Inst*. 2016;108(6):djv414.
8. Cha JH, Chan LC, Li CW, Hsu JL, Hung MC. Mechanisms controlling PD-L1 expression in cancer. *Mol Cell*. 2019;76(3):359-370.
9. Lee HT, Lee SH, Heo YS. Molecular interactions of antibody drugs targeting PD-1, PD-L1, and CTLA-4 in immuno-oncology. *Molecules*. 2019;24(6):1190.
10. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: mechanisms, response biomarkers, and combinations. *Sci Transl Med*. 2016;8(328):328rv324.
11. Sceneay J, Goreczny GJ, Wilson K, et al. Interferon signaling is diminished with age and is associated with immune checkpoint blockade efficacy in triple-negative breast cancer. *Cancer Discov*. 2019;9(9):1208-1227.
12. Li Z, Qiu Y, Lu W, Jiang Y, Wang J. Immunotherapeutic interventions of triple negative breast cancer. *J Transl Med*. 2018;16(1):147.
13. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and Nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med*. 2018;379(22):2108-2121.
14. Li CW, Lim SO, Xia W, et al. Glycosylation and stabilization of programmed death ligand-1 suppresses T-cell activity. *Nat Commun*. 2016;7:12632.
15. Lim SO, Li CW, Xia W, et al. Deubiquitination and Stabilization of PD-L1 by CSN5. *Cancer Cell*. 2016;30:925-939.
16. Cha J-H, Yang W-H, Xia W, et al. Metformin promotes antitumor immunity via endoplasmic-reticulum-associated degradation of PD-L1. *Mol Cell*. 2018;71(4):606-620.e607.

17. Li CW, Lim SO, Chung EM, et al. Eradication of triple-negative breast cancer cells by targeting glycosylated PD-L1. *Cancer Cell*. 2018;33(2):187-201.e110.
18. Melero I, Shuford WW, Newby SA, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nature Med*. 1997;3(6):682-685.
19. Weinberg AD, Morris NP, Kovacsovics-Bankowski M, Urba WJ, Curti BD. Science gone translational: the OX40 agonist story. *Immunol Rev*. 2011;244(1):218-231.
20. Zappasodi R, Sirard C, Li Y, et al. Rational design of anti-GITR-based combination immunotherapy. *Nature Med*. 2019;25(5):759-766.
21. Pollok KE, Kim YJ, Zhou Z, et al. Inducible T cell antigen 4-1BB. Analysis of expression and function. *J Immunol*. 1993;150(3):771-781.
22. Shuford WW, Klussman K, Tritchler DD, et al. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J Exp Med*. 1997;186(1):47-55.
23. Vinay DS, Kwon BS. 4-1BB signaling beyond T cells. *Cell Mol Immunol*. 2011;8(4):281-284.
24. Chester C, Sanmamed MF, Wang J, Melero I. Immunotherapy targeting 4-1BB: mechanistic rationale, clinical results, and future strategies. *Blood*. 2018;131(1):49-57.
25. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12(4):253-268.
26. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol*. 2007;179(2):977-983.
27. Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science*. 2001;291(5512):2364-2369.
28. Dwarakanath B, Singh D, Banerji A, et al. Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. *J Cancer Res Ther*. 2009;5(Suppl 1):S21-S26.
29. Shao B, Li CW, Lim SO, et al. Deglycosylation of PD-L1 by 2-deoxyglucose reverses PARP inhibitor-induced immunosuppression in triple-negative breast cancer. *Am J Cancer Res*. 2018;8(9):1837-1846.
30. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nat Rev Drug Discov*. 2011;10(9):671-684.
31. Lim SO, Li CW, Xia W, et al. EGFR signaling enhances aerobic glycolysis in triple-negative breast cancer cells to promote tumor growth and immune escape. *Cancer Res*. 2016;76(5):1284-1296.
32. Lee HH, Wang YN, Xia W, et al. Removal of N-linked glycosylation enhances PD-L1 detection and predicts anti-PD-1/PD-L1 therapeutic efficacy. *Cancer Cell*. 2019;36(2):168-178.e164.
33. Bartkowiak T, Jaiswal AR, Ager CR, et al. Activation of 4-1BB on liver myeloid cells triggers hepatitis via an interleukin-27-dependent pathway. *Clin Cancer Res*. 2018;24(5):1138-1151.
34. Woroniecka KI, Rhodin KE, Dechant C, et al. 4-1BB agonism averts TIL exhaustion and licenses PD-1 blockade in glioblastoma and other intracranial cancers. *Clin Cancer Res*. 2019. https://doi.org/10.1158/1078-0432.CCR-19-1068

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Kim B, Sun R, Oh W, Kim AMJ, Schwarz JR, Lim S-O. Saccharide analog, 2-deoxy-D-glucose enhances 4-1BB-mediated antitumor immunity via PD-L1 deglycosylation. *Molecular Carcinogenesis*. 2020;1–10. https://doi.org/10.1002/mc.23170