

Impact of PD-L1 upregulation on immune checkpoint inhibitor efficacy in triple-negative breast cancer using a 4T1 murine model

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Abstract. Triple-negative breast cancer (TNBC) is a lethal subtype of breast cancer with a poor prognosis and limited existing treatment options. The immune checkpoint inhibitor, anti-programmed death ligand 1 (PD-L1), has recently emerged as a promising alternative in treating TNBC. PD-L1 is critical in tumor immune evasion and is therefore a key target for cancer immunotherapy. Although anti-PD-L1 therapy is effective in breast cancer based on clinical trials, the relationship between PD-L1 expression levels and treatment response remains unclear. To investigate this, a 4T1 breast cancer cell line that stably overexpressed PD-L1 was established and was used to create a tumor model in mice. Mice were treated with anti-PD-L1 antibodies, and tumor growth was compared between the control and treated groups. PD-L1 overexpressing tumors did not exhibit an antitumor response to anti-PD-L1 therapy compared with the control tumors. Additionally, immune cell infiltration and activation were significantly altered, as shown by immunohistochemical staining and bulk RNA sequencing. In PD-L1-overexpressing tumors that did not respond to treatment, immune cell markers and antitumor immune pathways were downregulated. These results demonstrated that excessive PD-L1 expression creates an immunosuppressive tumor microenvironment, which impairs the efficacy of anti-PD-L1 therapy. The present

study suggests that excessive PD-L1 expression reduces the effectiveness of antitumor immunotherapy, and that PD-L1 expression levels are essential in predicting the response to antitumor immunotherapy.

Introduction

Programmed death ligand 1 (PD-L1), also known as CD274 or B7-H1, is a transmembrane protein expressed on the tumor surface that is a ligand for programmed cell death protein 1 (PD-1), which plays a critical role in immune escape mechanisms (1). PD-L1 on tumors binds to PD-1, a receptor on activated T cells, inhibiting T cell activation and causing immune evasion that neutralizes immune surveillance function against tumor cells (2,3). Immune checkpoint inhibitors (ICIs), particularly PD-1 and PD-L1 inhibitors, target immune evasion functions triggered by the binding of ligands and receptors on tumor and immune cells and have exhibited clinical efficacy against breast cancer and various solid malignancies (4). PD-L1 inhibitors provide a novel therapeutic paradigm by blocking immune evasion to enhance antitumor immunity (5).

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks an estrogen receptor, a progesterone receptor and HER2/neu (6). Patients with TNBC have a short overall survival time compared with other breast cancer subtypes; it is very aggressive and has a high rate of distant metastasis (6). TNBC is treated with chemotherapy as it cannot be treated with targeted therapy. Even with the application of these standard treatments, there are limits to improving the progression-free survival and overall survival rates; thus, new treatment strategies are needed for patients with TNBC (7). To overcome these limitations, the application of ICIs in recent years has made significant progress as a new treatment type for TNBC (8). Compared with other subtypes, TNBC has higher levels of PD-L1 expression and more immune cell infiltration in the tumor microenvironment (TME), making it a suitable candidate for ICI application (9). However, whether ICI

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therapy targeting PD-L1 improves the efficacy of TNBC treatment in actual clinical trials is controversial (10). While some studies suggest that a higher PD-L1 expression level correlates with improved therapeutic outcomes (11-13), other evidence indicates that excessive PD-L1 expression may contribute to therapeutic resistance (14).

The presence of tumor-infiltrating lymphocytes (TILs) in the TME is an essential factor in predicting the response to immunotherapy (15). Immune-high tumors exhibit high expression of TILs and PD-L1, which has provided a basis for the application of ICIs in TNBC (16,17). Types of TILs include CD8⁺ T cells, CD4⁺ T cells, B cells, natural killer (NK) cells and macrophages (18,19). High levels of TILs have been associated with improved responses to ICIs, as they enhance the antitumor immune response (20).

Anti-PD-L1, used in therapy for targeting PD-L1, is associated with a complex relationship between PD-L1 expression and the TME in TNBC. Therefore, the present study investigated whether the difference in the efficacy of anti-PD-L1 treatment is caused by the intra-tumoral expression of PD-L1. To understand the cause of the difference, the directionality of the genes related to the tumor infiltration of immune cells were investigated through bulk RNA sequencing. Based on these results, it was explored whether the difference in the TME according to the expression of PD-L1 affects the therapeutic efficacy of anti-PD-L1.

Materials and methods

PD-L1 expression and survival analysis in clinical data of breast cancer cohorts. In the present study, two publicly available datasets, namely the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort and The Cancer Genome Atlas (TCGA)-BRCA provisional cohort, were used. The METABRIC dataset on breast cancer (21,22) and the TCGA dataset on breast invasive carcinoma [TCGA, PanCancer Atlas (23)] were obtained from cBioportal (<https://www.cbioportal.org/>) in August 2023. PD-L1 expression analysis was conducted on the METABRIC (total n=1,905, basal n=296) and TCGA (total n=1,084, basal n=171) datasets according to subtype and TN [tumor size (T) and lymph node status (N)] stage. Subsequently, survival analysis was performed on patients with TNBC (METABRIC, n=233; TCGA, n=163) by dividing them into high and low-PD-L1 expression groups. PD-L1 mRNA expression was quantified using z-scores, which represent the number of standard deviations a value is from the mean expression across all samples. Patients were classified as high (z-score ≥ 0) or low (z-score < 0) PD-L1 expression accordingly. Overall survival was estimated using the Kaplan-Meier method, and statistical differences between groups were assessed using the log-rank test.

Cell lines and culture. The mouse TNBC cell line, 4T1 [cat. no. CRL-2539; American Type Culture Collection (ATCC®)], and the mouse colon carcinoma cell line, CT26 (cat. no. CRL-2638; ATCC®), were used in this study. CT26 cells were used as a source of mouse wild-type PD-L1 cDNA, which was amplified by PCR for subsequent cloning and overexpression in 4T1 cells. The cells were cultured in RPMI 1640 medium (Welgene, Inc.) and Dulbecco's Modified Eagle's

Medium (Welgene, Inc.) supplemented with 10% fetal bovine serum (FBS; Welgene, Inc.) and 1% penicillin-streptomycin (10,000 U/ml; Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Establishment of an PD-L1 overexpressing stable cell line. The murine colorectal carcinoma cancer cell, CT26, derived from BALB/C mice, was used to establish the PD-L1 overexpressing stable cell line. Mouse PD-L1 RNA was extracted from the CT26 cells with TRIzol (FAVORGEN Biotech Corp.), and the mouse PD-L1 gene (RefSeq: NM_021893.3) was amplified through reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was performed using PrimeScript™ RT Reagent Kit (cat. no. RR037A; Takara Bio, Inc.) according to the manufacturer's instructions. The following two primers, incorporating a Kozak sequence for efficient translation and *NheI* and *NotI* restriction sites for cloning, were used: 5'-GCTAGCGCCACCATGAGGATA TTTGCTGGCATT-3' (forward) and 5'-GCGGCCGCTTAC GTCTCCTCGAATTGTGT-3' (reverse). The PCR product was ligated into the pMD20-T vector (Takara Korea Biomedical Inc.) to create restriction enzyme sites. The pMD20-T vector was transformed into *Escherichia coli* DH5 α competent cells (cat. no. CP011; Enzymomics, Inc.). The amplified PD-L1 gene with restriction enzyme sites was inserted using the *NheI* and *NotI* sites in a PiggyBac vector (System Biosciences, LLC). The constructed PiggyBac vector containing the mouse PD-L1 gene and an empty PiggyBac control vector were transfected into 4T1 cells using JetPrime (Polyplus-transfection SA). Transfection was performed in 100 mm dishes using 10 μ g of plasmid DNA per dish at 37°C for 24 h, according to the manufacturer's instructions. After transfection, stable PD-L1 overexpressing cell lines were established by treating the cells with 5 μ g/ml puromycin starting 48 h later. Puromycin was used at 5 μ g/ml for selection and 1 μ g/ml for maintenance in both PD-L1-overexpressing and control (empty PiggyBac vector) 4T1 cells. Following antibiotic selection, GFP-positive cells were collected and PD-L1 overexpression was confirmed by both surface staining using APC-conjugated anti-mouse PD-L1 antibody (1:100; cat. no. 124312; BioLegend, Inc.) and western blot analysis with anti-PD-L1 antibody (1:1,000; cat. no. 60475; Cell Signaling Technology, Inc.). To isolate a subpopulation of cells with intermediate PD-L1 expression, cells were subsequently sorted using a FACSaria III cell sorter (BD Biosciences) operated with FACSDiva software (version 9.2; BD Biosciences), based on GFP and PD-L1 fluorescence intensity. For surface PD-L1 staining, cells were incubated with APC-conjugated anti-mouse PD-L1 antibody (1:100; cat. no. 124312; BioLegend, Inc.) in FACS buffer (PBS containing 2% FBS) for 30 min at 4°C in the dark. After staining, cells were washed twice with PBS and filtered through a 40 μ m cell strainer prior to sorting. Cells were gated into subpopulations with low, medium or high PD-L1 expression levels based on GFP and PD-L1 fluorescence intensity. The sorted sublines were subsequently expanded, and PD-L1 expression levels were validated by western blot analysis using an anti-PD-L1 antibody (1:1,000; cat. no. 60475; Cell Signaling Technology, Inc.) as described below. For experimental consistency, the 4T1 control population with no

PD-L1 overexpression was designated as 4T1-Ctl, the PD-L1 overexpression medium-expression group as 4T1-PD-L1 O/E (Medium level) and the highest-expression group as 4T1-PD-L1 O/E.

Immunoblotting. Cells were lysed in RIPA buffer (MilliporeSigma) mixed with a protease inhibitor cocktail (Thermo Fisher Scientific, Inc.) and 0.5 M Tris-EDTA (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), and 20 μ g of total protein was loaded per lane. The prepared proteins were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto Immobilon-P transfer membranes (Merck KGaA). Membranes were blocked with 5% skim milk in TBST (TBS containing 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated with the following primary antibodies overnight at 4°C: Anti-PD-L1 (1:1,000; cat. no. 60475, Cell Signaling Technology, Inc.) and anti- β -actin (1:5,000; cat. no. sc-4778, Santa Cruz Biotechnology, Inc.). After washing, membranes were incubated with the following HRP-conjugated secondary antibodies for 1 h at room temperature: Goat anti-rabbit IgG-HRP (1:5,000; cat. no. sc-2004, Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG-HRP (1:5,000; cat. no. sc-2005, Santa Cruz Biotechnology, Inc.). After membrane incubation with specific antibodies, the signal was enhanced with chemiluminescence reagents (Thermo Fisher Scientific, Inc.) and measured using an Amersham Imager 680 (GE Healthcare). The relative values of the bands observed by western blotting were analyzed using ImageJ software (version 1.53j; National Institutes of Health).

RT-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using the Tri-RNA reagent (FAVORGEN Biotech Corp.). cDNA was synthesized from the extracted RNA using the PrimeScript™ RT Reagent kit (cat. no. RR037A; Takara Korea Biomedical Inc.; Takara Bio Inc.), according to the manufacturer's instructions. qPCR was conducted on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green PCR master mix (cat. no. 4367659; Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The relative mRNA levels were measured using the $2^{-\Delta\Delta C_q}$ method (24), with *Gapdh* as the control. The following primer sequences were used: *Pd-L1*, (forward) 5'-GCGGACTACAAGCGAATCACG-3' and (reverse) 5'-CTCAGCTTCTGGATAACCCTCG-3'; *Gapdh*, (forward) 5'-CATCACTGCCACCCAGAAGACTG-3' and (reverse), 5'-ATGCCAGTGAGCTTCCCGTTTCAG-3'.

Flow cytometry. The cells were harvested using 0.25% trypsin and washed with PBS. The prepared cells were suspended in 1% BSA or PBS. Staining was conducted using a mouse PD-L1 antibody conjugated to phycoerythrin (cat. no. 124307; BioLegend, Inc.) and 7-AAD (cat. no. 00-6993-50; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at room temperature in the dark. The mean fluorescence intensity was detected using a BD FACSCanto™ Flow Cytometer (BD Bioscience),

and the data were analyzed using BD FACSDiva software (version 8.0.1; BD Biosciences; <https://www.bdbiosciences.com/>).

Migration, invasion and gap closure assays. For migration assays, 1×10^5 cells were seeded in an 8.0- μ m pore size insert (Falcon®; Corning Life Sciences) with serum-free medium. For invasion assessment, an insert pre-coated with 1 mg/ml Matrigel (by incubation for 20 min at 37°C) was used, with 1×10^5 cells in serum-free medium. A medium containing 10% FBS was added to the lower chambers. After incubating for 12 h at 37°C, cells that migrated were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with a 0.1% crystal violet for 2 min at room temperature. Stained cells were imaged using a light microscope equipped with a CCD camera (Leica Microsystems, Inc.) and quantified with ImageJ software.

For the gap closure assay, 5×10^4 cells were seeded in a culture-insert in a 2-well μ -dish (Ibidi GmbH) and incubated overnight at 37°C. After reaching confluency, a gap was made by removing the insert, the plate was washed with PBS and 500 μ l medium containing 10% FBS (25) was added. Gap closure was observed from 0 to 12 h using a light microscope equipped with a CCD camera (Leica Microsystems, Inc.).

Cell proliferation assay. The CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega Corporation) was used to assess cell proliferation. A total of 3×10^3 cells were seeded into each well of a 96-well plate and incubated at 37°C for 24, 48 and 72 h. Afterward, 100 μ l of the CellTiter-Glo solution was added to the cells following the manufacturer's guidelines. Luminescence was measured using a Luminiskant ascent (Thermo Fisher Scientific, Inc.).

Analysis of immune cell populations. Tumors were digested in collagenase and hyaluronidase for 30 min in a 37°C shaking incubator, followed by an RBC lysis buffer treatment (Thermo Fisher Scientific, Inc.). The cells were filtered through 40- and 75- μ m cell strainers, and 1×10^6 cells were suspended in the staining buffer (BioLegend, Inc.). Cells were pre-incubated with 0.25 μ g of the TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (BioLegend, Inc.) for 5-10 min. Antibodies were then incubated on ice for 20 min in the dark. Fluorescence intensity was detected using a BD FACSCanto™ Flow Cytometer (BD Bioscience), and the data were analyzed using BD FACSDiva software (version 8.0.1; BD Biosciences; <https://www.bdbiosciences.com/>). The following antibodies from BioLegend, Inc. were used for lymphoid panel staining: PE anti-mouse CD3 (cat. no. 100206), Brilliant Violet 421 anti-mouse CD4 antibody (cat. no. 100437), Brilliant Violet 510 anti-mouse CD45 antibody (cat. no. 103137) and PerCP/Cyanine5.5 anti-mouse CD8a antibody (cat. no. 100733).

Syngeneic mouse model and antibody treatment. *In vivo* experiments were conducted at the animal facility of Seoul National University Hospital (Seoul, South Korea), following guidelines and obtaining prior approval from the Institutional Animal Care and Use Committee (IACUC; approval no. 23-0157-S1A0). Animals were maintained in the facility-accredited AAALAC International (no. 001169) in

accordance with Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2010). In total, 54 6-week-old female BALB/c mice (body weight, 18–22 g) were obtained from Koatech. Mice were housed under specific pathogen-free conditions at 22±2°C with 55±10% humidity, under a 12 h light/dark cycle, with *ad libitum* access to food and water. Animal health and behavior were monitored daily. Syngeneic mouse models were established by injecting 1×10⁵ control 4T1, PD-L1-overexpressing 4T1 (medium level), or PD-L1 overexpressed 4T1 cells into the mammary gland fat pads. Tumor size was measured twice weekly using digital calipers and calculated with a modified ellipsoidal formula [volume=1/2 (length × width²)]. In this experiment, two groups of 4T1 tumor-bearing mice were used: One injected with control 4T1 cells and the other with 4T1 cells overexpressing PD-L1 at a high level. In a subsequent analysis, an additional group bearing 4T1 tumors with moderate PD-L1 overexpression was included, resulting in three experimental groups: Control, medium PD-L1 expression and high PD-L1 expression. Each group was treated with either control IgG or anti-PD-L1. The PD-L1 inhibitor, a monoclonal anti-PD-L1 antibody (cat. no. BP0101; 150 µg/100 µl) and immunoglobulin G (IgG; cat. no. BP0090; 150 µg/100 µl) were administered intraperitoneally a total of three times, starting when tumor volumes reached ~100 mm³. The experiments were concluded after three intraperitoneal injections of either IgG or anti-PD-L1, on days 6, 9 and 12. Additionally, any mouse with a tumor volume reaching 1,000 mm³ was scheduled for immediate euthanasia. The study was designed with an appropriate sample size to account for biological variability in accordance with IACUC guidelines, and the animal was included in the analysis. The experiments lasted for a total of 17 or 13 days, with the shorter duration resulting from earlier tumor progression in some mice necessitating humane endpoint euthanasia (tumor volume reached 1,000 mm³). At the end of the study, mice were euthanized following deep anesthesia using isoflurane (2–5% for induction, 1–3% for maintenance). CO₂ inhalation was used to complete euthanasia at a displacement rate of 30–70% of the chamber volume per minute, following NIH and AVMA (2020) guidelines. Death was confirmed by the cessation of respiration, which was monitored continuously for at least 10 min following CO₂ exposure to ensure complete loss of vital signs. After euthanasia, tumors, lungs and spleens were resected and then preserved in 4% paraformaldehyde at 4°C overnight or Bouin's solution at room temperature overnight for further analysis.

Immunocytochemistry. Control and PD-L1-overexpressing 4T1 murine breast cancer cells were seeded on 8-well chamber slides (cat. no. 154534; Nunc; Thermo Fisher Scientific, Inc.) and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% Tween-20 in PBS for 10 min. Non-specific binding was blocked with 1% BSA (cat. no. AC1025-100-00; Biosesang) in PBS for 1 h at room temperature. Cells were incubated overnight at 4°C with a fluorescence-conjugated anti-PD-L1 antibody (1:200; cat. no. MAB90782AF647; Novus Biologicals, LLC) diluted

in blocking buffer. The next day, cells were washed three times with PBS. Nuclei were counterstained with DAPI (1 µg/ml; cat. no. S7113; Merck KGaA) for 5 min at room temperature. Slides were mounted using fluorescence mounting medium (cat. no. S3023; Dako; Agilent Technologies), and representative images were captured using a fluorescence microscope (ECLIPSE Ni-E; Nikon Corporation).

Immunohistochemistry. The primary tumors were fixed in 4% buffered paraformaldehyde at 4°C overnight, embedded in paraffin and sectioned into 4-µm slices. After being deparaffinized in xylene and rehydrated in graded ethanol and water solutions, antigen retrieval was performed using citrate (pH 6.0) or 10 mM Tris/1 mM EDTA (pH 9.0) at 95°C for 20 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 20 min at room temperature. The sections were then blocked with 10% normal goat serum (cat. no. S-1000; Vector Laboratories, Inc.) for 1 h at room temperature to prevent non-specific binding, followed by overnight incubation at 4°C with primary antibodies. The antibodies used were as follows: CD45 (1:100; cat. no. AF114; R&D Systems, Inc.), CD4 (1:200; cat. no. ab183685; Abcam), CD8 (1:200; cat. no. ab209775; Abcam) or PD-L1 (rabbit polyclonal; 1:200; cat. no. ab10558; Abcam). After washing, the sections were incubated with species-appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. For rabbit and mouse primary antibodies, a ready-to-use EnVision+ System-HRP (Rabbit/Mouse) (cat. no. K5007; Dako; Agilent Technologies) was used. For the goat-derived CD45 antibody, rabbit anti-goat IgG(H+L)-HRP (1:500; cat. no. SA007-500; GenDEPOT, LLC) was used. Immunoreaction was visualized using a DAB chromogen kit included in the EnVision system (cat. no. K5007; Dako; Agilent Technologies, Inc.). Nuclei were counterstained with hematoxylin (cat. no. S330930-2; Dako; Agilent Technologies, Inc.) for 2 min at room temperature. Histological images were captured using a light microscope equipped with a CCD camera (Leica Microsystems, Inc.).

Identification of differentially expressed genes (DEGs) and functional enrichment analysis. RNA was extracted from tumors with confirmed PD-L1 inhibitory effects, and transcriptome sequencing was performed by Macrogen, Inc. (Seoul, Korea). RNA quality and integrity were assessed using the 2100 Bioanalyzer System (cat. no. G2939BA, Agilent Technologies, Inc.) with a DNA 1000 Kit (cat. no. 5067-1504, Agilent Technologies, Inc.). The SureSelectXT RNA Direct Library Preparation Kit (cat. no. G7550A; Agilent Technologies, Inc.) was used for library preparation, followed by quality control of the raw sequencing reads. Sequencing was carried out on the NovaSeq 6000 platform (cat. no. 20012850, Illumina, Inc.) using paired-end 101-bp reads. Raw sequencing data were first evaluated using FastQC (version 0.11.7; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) to assess read quality. Adapter sequences and low-quality bases were trimmed using Trimmomatic (version 0.38; <http://www.usadellab.org/cms/?page=trimmomatic>). The cleaned reads were aligned to the *Mus musculus* reference genome (mm10) using HISAT2 (version 2.1.0; <https://ccb.jhu.edu/software/hisat2/>), with internal use of Bowtie2 (version 2.3.4.1). Transcript assembly was performed using StringTie (version 2.1.3b; <https://ccb.jhu.edu>).

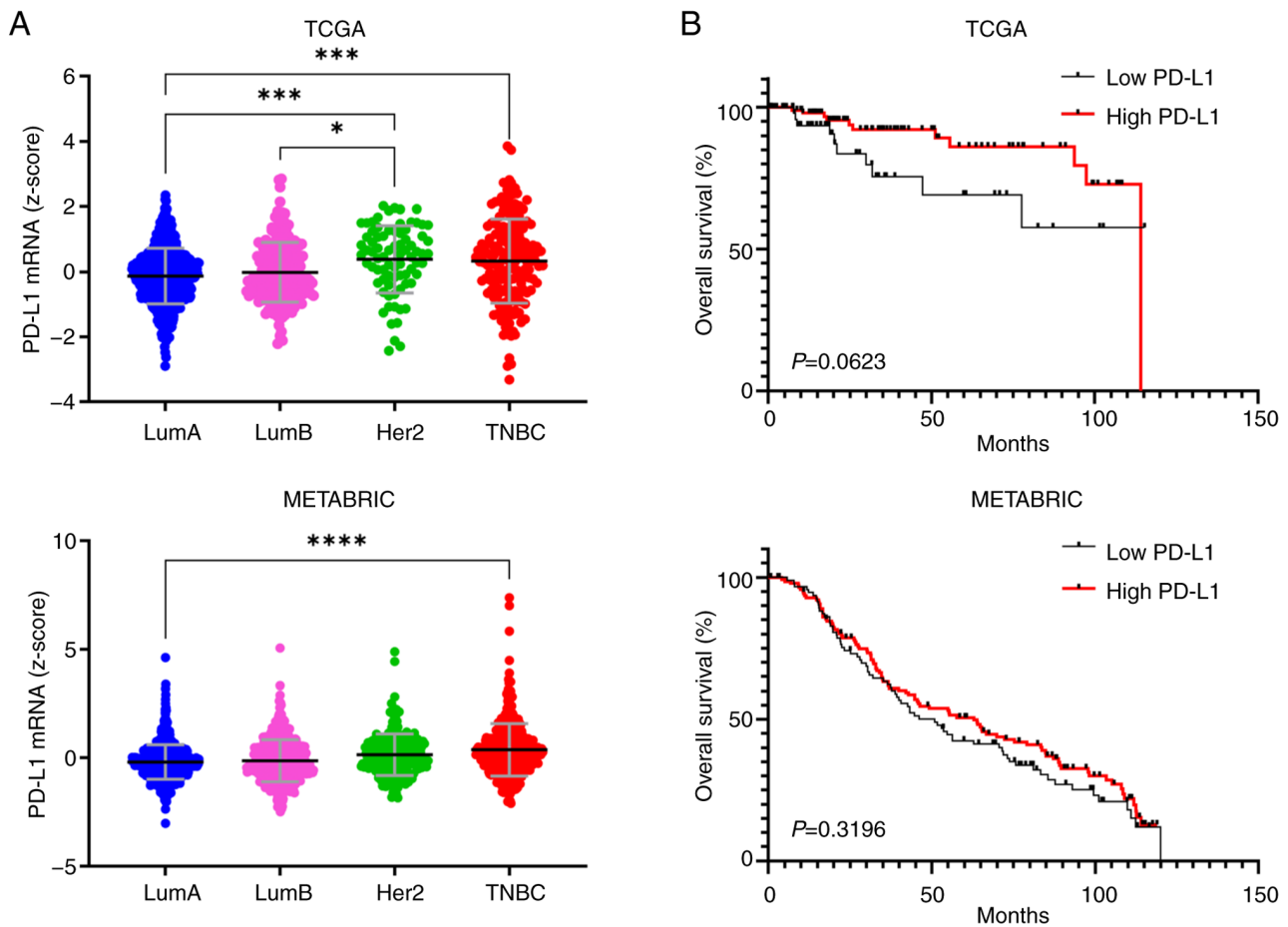


Figure 1. PD-L1 expression and survival analysis in the TCGA and METABRIC datasets. (A) Expression levels of PD-L1 in breast cancer subtypes (TCGA, n=1,084; METABRIC, n=1,905). (B) Kaplan-Meier curves according to PD-L1 expression in TNBC (TCGA, n=163; METABRIC, n=233; two-sided log-rank test). * $P<0.05$, *** $P<0.001$, **** $P<0.0001$ by one-way ANOVA with Tukey's post hoc test. PD-L1, programmed death-ligand 1; TCGA, The Cancer Genome Atlas; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TNBC, triple-negative breast cancer; LumA/B, Luminal A and Luminal B subtypes.

edu/software/stringtie/). Differential expression analysis was conducted using DESeq2 (version 1.36.0; <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>). Expression profiles were generated, and DEGs were identified based on a $\text{fold-change} > 2$ and raw $P < 0.05$. The P-values were adjusted for multiple testing using the Benjamini-Hochberg method, with a false discovery rate threshold of 0.05. DEGs were then functionally annotated using the Database for Annotation, Visualization and Integrated Discovery (<https://david.ncifcrf.gov>). Gene Ontology (<https://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<https://www.genome.jp/kegg/>) analyses were subsequently performed.

Statistical analysis. To evaluate the data, graphs were constructed to display the mean $\pm$ standard deviation derived from a minimum of three independent experiments. Statistical comparisons between two groups were performed using unpaired t-tests and the Mann-Whitney U test. For experiments involving two independent variables (such as treatment, expression status or time), data were analyzed using two-way ANOVA followed by Tukey's post hoc test. In survival analysis, the Kaplan-Meier method with a two-sided log-rank test was used. Statistical analyses were performed using GraphPad

Prism v9.2.0 (Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Survival rate of patients with TNBC according to PD-L1 expression and clinicopathologic characteristics. To understand the relationship between PD-L1 expression and clinicopathology in breast cancer subtypes, TCGA and METABRIC datasets were analyzed. It was observed that TNBC had a high level of PD-L1 expression in both TCGA and METABRIC datasets, similar to the HER2 subtype in TCGA dataset (Fig. 1A). The difference in overall survival between patients with TNBC divided into the high and low PD-L1 mRNA expression groups in the TCGA and METABRIC datasets was not statistically significant (Fig. 1B). Similarly, PD-L1 expression was not significantly associated with T stage and nodal involvement (Fig. S1). These results confirm that, although PD-L1 expression is elevated in TNBC, it may not be directly associated with poor patient prognosis based on public datasets.

PD-L1 overexpression and its impact on 4T1 breast cancer cells. To further elucidate the role of PD-L1 upregulation in breast cancer, a 4T1 murine breast cancer cell line that stably

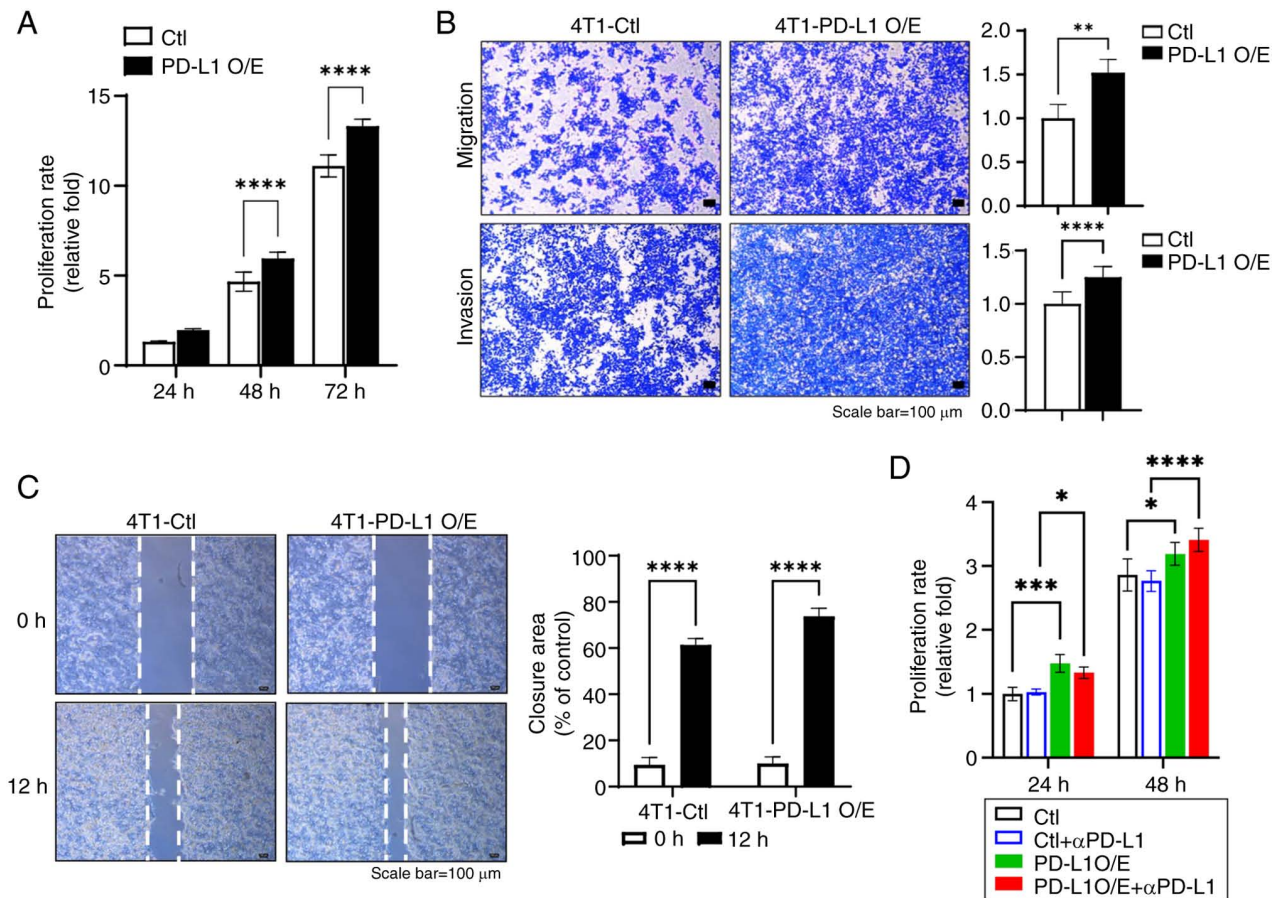


Figure 2. Effect of PD-L1 overexpression on phenotypic changes and the impact of α PD-L1 treatment on proliferation. (A) Relationship between the proliferation rate and overexpression of PD-L1 in the 4T1 cell line ($n=3$, mean $\pm$ SEM; **** $P<0.0001$ by two-way ANOVA with Tukey's post hoc test). (B) Images and quantification data examined in the migration and invasion of PD-L1-overexpressed 4T1 cells ($n=3$, mean $\pm$ SEM; ** $P<0.01$ and **** $P<0.0001$ by Mann-Whitney U test). (C) Images and quantification data examined in the gap closure assay of PD-L1-overexpressed 4T1 cells ($n=3$, mean $\pm$ SEM; **** $P<0.0001$ two-way ANOVA with Tukey's post hoc test). (D) Proliferation rate in response to α PD-L1 treatment in 4T1 cells with PD-L1 overexpression ($n=3$, mean $\pm$ SEM; * $P<0.05$, **** $P<0.0001$ by two-way ANOVA with Tukey's post hoc test). α PD-L1, anti-PD-L1; PD-L1, programmed death-ligand 1; O/E, overexpression; Ctl, control.

overexpressed PD-L1 was established. PD-L1 overexpression was confirmed at the protein and mRNA levels in the 4T1 PD-L1 overexpressed cell line (Fig. S2A and B). The cell surface expression of PD-L1 was further validated using flow cytometry and immunocytochemistry (Fig. S2C and D). To determine the phenotypic importance of PD-L1-overexpression, the proliferation, migration and invasion of the cells was examined. The result showed that PD-L1 O/E induced enhanced the proliferative capacity of 4T1 cells compared with the control cells (Fig. 2A). Additionally, the overexpression of PD-L1 led to significant increases in the migratory and invasive capabilities, as evidenced by the migration, invasion and gap closure assays (Fig. 2B and C). To investigate the therapeutic implications of PD-L1 overexpression, PD-L1-overexpressing 4T1 cells were treated with anti-PD-L1 antibodies. The effects of anti-PD-L1 treatment were not pronounced, with only modest changes in cell proliferation observed. Specifically, there was a slight reduction in proliferation at 24 h, followed by an increase at 48 h, though these changes were not statistically significant (Fig. 2D). This suggests that the interaction between PD-L1 signaling and tumor cell survival mechanisms may be complex, potentially involving compensatory pathways that mitigate the effects of PD-L1 blockade.

PD-L1 expression is crucial for the antitumor efficacy of anti-PD-L1 therapy in TNBC. ICIs targeting PD-L1 could be applied when the PD-L1 expression level on tumor or immune cells in the tumor tissue is above a specific value. Most clinical benefits occur in patients with high levels of PD-L1 expression (12). However, not all patients with high PD-L1 levels show consistent treatment effects, suggesting that the relationship between PD-L1 expression and treatment efficacy is not yet fully understood (12). To further investigate the efficacy of anti-PD-L1 depending on PD-L1 expression level, 4T1 breast cancer cell lines with different PD-L1 expression levels were established and transplanted into syngeneic breast tumor mouse models to observe the effect of anti-PD-L1 on tumors. It was observed that anti-PD-L1 treatment had no antitumor effect on tumors generated from 4T1-PD-L1 O/E cell lines. In the 4T1-Ctl group with a very low PD-L1 expression level, an antitumor effect of anti-PD-L1 (Fig. 3A and B) was observed. Tumor weight measurements confirmed that PD-L1 overexpression reduced the anti-PD-L1 antitumor effect compared with the 4T1-Ctl group (Fig. 3C). Notably, in the control + anti-PD-L1 group, 1 animal died on day 10 of the study. Necropsy revealed no apparent abnormalities, and the animal was included in the analysis. Compared with tumors derived

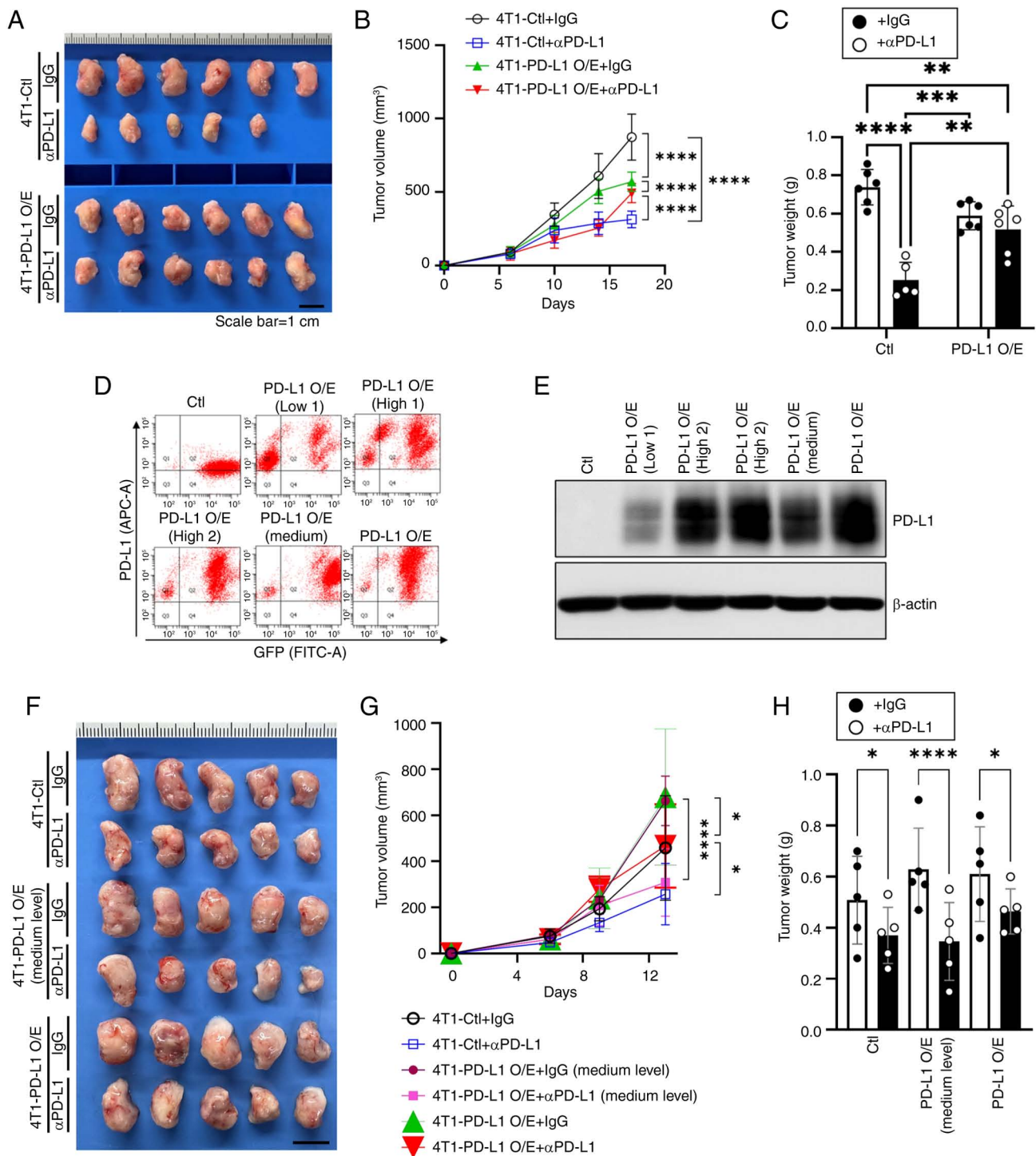


Figure 3. Differential effectiveness of αPD-L1 treatment on 4T1 breast tumors depending on the PD-L1 expression levels. (A) Images of the tumor tissues (scale bar, 1 cm) and the (B) volume and (C) weight of tumors treated with IgG or αPD-L1 with or without high levels of PD-L1 overexpression (n=6, mean ± SEM; **P<0.01, ***P<0.001, ****P<0.0001 by two-way ANOVA with Tukey's post hoc test). (D) Flow cytometric sorting of 4T1 cells based on GFP expression and PD-L1 levels and (E) immunoblot validation of PD-L1 expression in the sorted cell lines. (F) Images of tumor tissues (scale bar, 1 cm) and the tumor (G) volume and (H) weight of mice treated with IgG or αPD-L1 with or without medium or high levels of PD-L1 overexpression (n=5, mean ± SEM; *P<0.05, ****P<0.0001 by multiple t-two-way ANOVA with Tukey's post hoc test). αPD-L1, anti-PD-L1. αPD-L1, anti-PD-L1 antibody; PD-L1, programmed death-ligand 1; IgG, immunoglobulin G; GFP, green fluorescent protein; Ctl, control; O/E, overexpression.

from 4T1 control cells with low PD-L1 expression, tumors with high PD-L1 expression exhibited a markedly weaker response to anti-PD-L1 treatment, indicating that PD-L1 overexpression reduced the antitumor efficacy of the therapy (Fig. 3A-C).

To clarify this observation, the anti-PD-L1 effect was further tested by additionally including a group with

medium-PD-L1-expression [PD-L1 O/E (Medium)], which had a lower PD-L1 expression level than the previously described overexpression group. The medium-level group was collected and confirmed by GFP sorting and immunoblotting (Fig. 3D and E). Of note, anti-PD-L1 treatment was most effective in the 4T1-PD-L1 O/E (Medium level) group and

the treatment outcomes were similar to those observed in the 4T1-Ctl group; in some tumors, treatment exhibited improved antitumor effects (Fig. 3F and G). The improved antitumor effect in the 4T1-PD-L1 O/E (Medium level) group was also reflected in the final tumor weight measurements (Fig. 3H).

These findings demonstrate that PD-L1 expression is essential in determining the efficacy of anti-PD-L1 therapies. Excessive PD-L1 levels diminish treatment response, while the medium-level exhibited the greatest sensitivity to tumor treatment. This suggests that there may be an optimal PD-L1 expression range to maximize the effect of anti-PD-L1 therapy. This research emphasizes the need to further refine patient selection criteria for anti-PD-L1 therapy, as PD-L1 expression levels above a certain level alone may not be sufficient to predict treatment success.

PD-L1 overexpression modulates immune cell infiltration and T cell activation following anti-PD-L1 treatment. PD-L1 expression and treatment responses are related to T cell infiltration into tumors (26). In this regard, based on the aforementioned results, it was evaluated whether there was a difference in immune cell infiltration according to anti-PD-L1 treatment between the control group and the PD-L1-overexpressing tumor group. It was first confirmed whether PD-L1 was expressed at a high level in tumors overexpressing PD-L1 (Fig. 4A). To understand the relationship between the anti-PD-L1 effect and immune cell infiltration, immunohistochemistry staining was performed for CD45, a total immune cell marker, on tumor sections. It was observed that the control tumors, in which anti-PD-L1 treatment exhibited a notable antitumor effect, were infiltrated with immune cells (Fig. 4B). By contrast, the PD-L1-overexpressing group, in which anti-PD-L1 treatment exhibited a weak antitumor effect, had fewer infiltrated immune cells than the control tumors (Fig. 4B). Additionally, high levels of immune cell infiltration were also observed in PD-L1 overexpressing tumors treated with IgG. This result suggests that PD-L1 overexpression was not related to total immune cell infiltration.

The group differences in the distribution of CD4⁺ and CD8⁺ T cells were analyzed, which are associated with the anti-PD-L1 antitumor effect among immune cells. In the control tumors, where anti-PD-L1 treatment was highly effective, infiltrated CD4⁺ and CD8⁺ T cells were more numerous than in the other groups (Fig. 4C). By contrast, tumors overexpressing PD-L1 exhibited only a slight increase in CD4⁺ and CD8⁺ T cell infiltration levels after anti-PD-L1 treatment compared with the control (Fig. 4C). Notably, in these tumors, anti-PD-L1 treatment lowered the infiltration of both types of T cells compared with the control anti-PD-L1 treatment group. For validation of the aforementioned results, flow cytometry was performed. The control group treated with anti-PD-L1 had higher CD4⁺ and CD8⁺ T cell infiltration levels, whereas the tumors treated with anti-PD-L1 that overexpressed PD-L1 had reduced T cell distribution (Figs. 4D and S3).

To understand the underlying mechanism for these observations, the tumor samples were subjected to bulk RNA sequencing and group specific DEGs were applied to the pathway analysis. Using the identified DEGs and pathways, the DEGs were compared between the control tumors treated with IgG and those treated with anti-PD-L1. This method

led to the identification of pathways specifically upregulated by anti-PD-L1, independent of PD-L1 overexpression. To assess downregulation in primary resistance, DEGs were also compared between control tumors and PD-L1-overexpressing tumors, both treated with anti-PD-L1. Analysis of the upregulated genes in the control + anti-PD-L1 group showed enhanced signaling related to T cell activation, with increased PD-L1 and PD-1 immune check point pathway activities, as well as upregulation of Th17 and Th1/Th2 cell differentiation KEGG pathways (Fig. 4E). By contrast, the PD-L1 O/E + anti-PD-L1 group exhibited significant downregulation of genes involved in T cell signaling, the PD-L1-PD-1 pathway and various immune cell-related signaling pathways, compared with the control treatment group (Fig. 4F). These results suggest that while PD-L1 upregulation contributes to immune cell infiltration within tumors, excessive PD-L1 levels may impair T cell activation and overall immune responses, thereby compromising the efficacy of anti-PD-L1 therapy. These findings highlight the importance of PD-L1 expression levels in determining the success of the immune checkpoint blockade and suggest that excessively high PD-L1 expression levels may lead to an immunosuppressive microenvironment that impedes the therapeutic effect of PD-L1 inhibitors.

Discussion

The present study focused on investigating the antitumor response to anti-PD-L1 therapy according to the expression level of PD-L1 and on determining the reasons for the difference in response. The application of ICIs to TNBC, which has limited treatment options due to the lack of clear targeted therapies, has emerged as a new treatment strategy, but not all patients respond well to the treatment (27). PD-L1, highly expressed in various cancer types and involved in immune evasion, is a crucial target of cancer immunotherapy, but its role and function according to the degree of expression are not yet known. Due to the potential implications of PD-L1 expression levels, it is essential to investigate whether these levels may limit the effectiveness of anti-PD-L1 therapy. In the present study, 4T1 cell lines with various levels of PD-L1 were transplanted into a breast cancer mouse tumor model, which were then treated with anti-PD-L1, providing evidence that there is a difference in the immunotherapy response depending on the expression level of PD-L1. The results support that the expression level of PD-L1 has a pivotal role in immunotherapy and that refined clinical strategies considering this are needed. PD-L1 is associated with the response to anti-PD-1 or anti-PD-L1 therapy in various cancer types (28-30). In the IMpaassion130 study that assessed the efficacy of atezolizumab (targets PD-L1), the PD-L1-positive patient subgroup exhibited prolonged overall survival and progression-free survival (12,31). By contrast, the GeparNuevo clinical trial of another anti-PD-L1 agent reported that durvalumab did not induce differences in therapeutic efficacy according to PD-L1 status (13). These studies suggest that the efficacy of ICIs according to the status of PD-L1 expression in cancer remains unclear. In clinical trials, PD-L1 levels in immune cells and tumor cells have been used as a criterion for selecting patient groups (20). Nevertheless, the efficacy of ICI therapy was not guaranteed in a number of patients with high tumor PD-L1 expression levels (32-35).

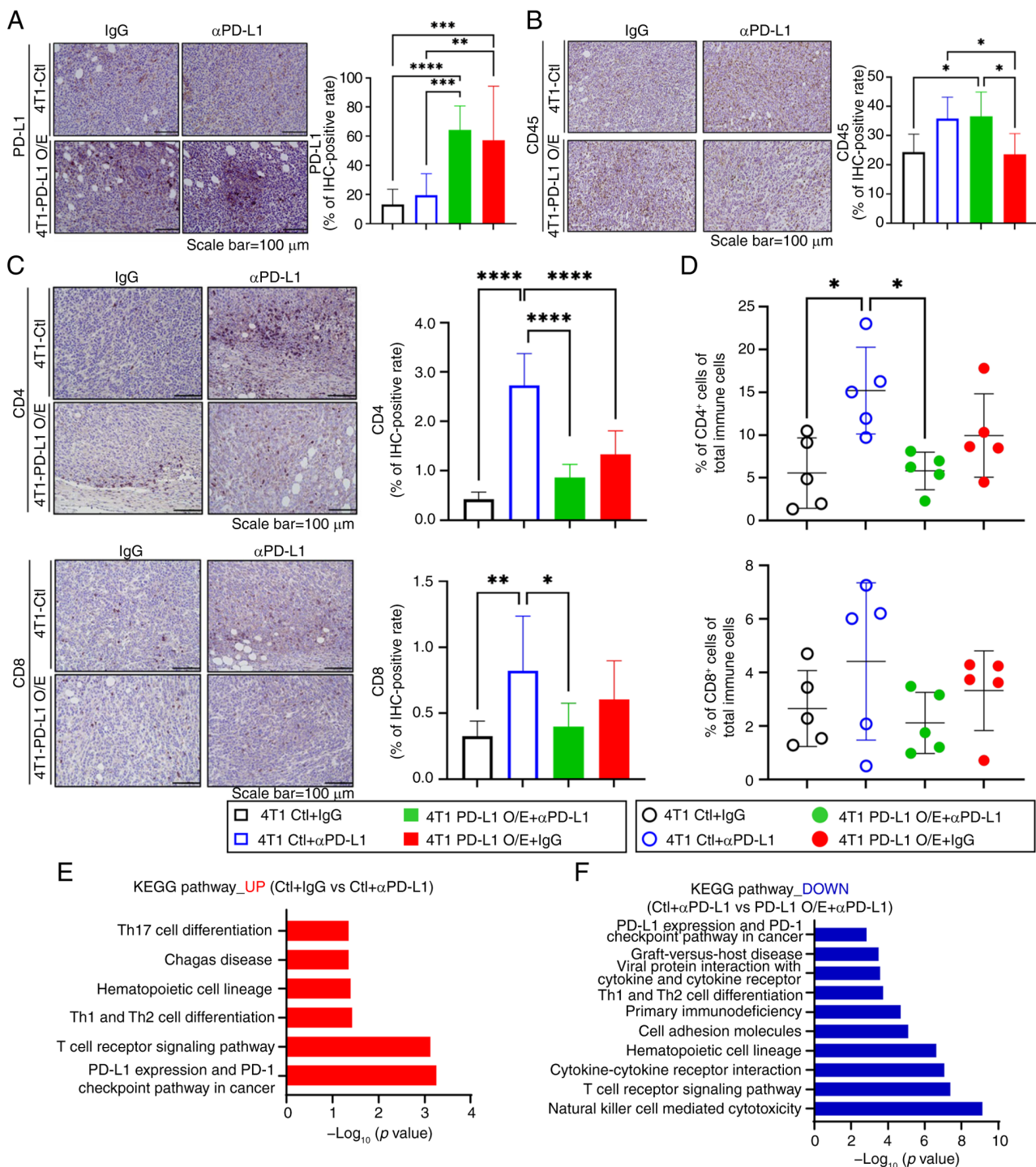


Figure 4. Influence of PD-L1 overexpression on immune cell infiltration and T cell activation in response to α PD-L1 therapy. Representative (A) PD-L1 and (B) CD45 immunohistochemistry images (scale bar, 100 μ m) and the corresponding quantification data of the tumor tissues (n=3, mean $\pm$ SEM; * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001 by two-way ANOVA with Tukey's post hoc test). (C) Representative tissue images of stained CD4 and CD8 (scale bar, 100 μ m) and the corresponding quantification data of the images (n=5, mean $\pm$ SEM; * P <0.05, ** P <0.01, **** P <0.0001 by two-way ANOVA with Tukey's post hoc test). (D) Percentage of CD4 and CD8⁺ T cells in the total immune cells analyzed through flow cytometry (n=5, mean $\pm$ SEM; * P <0.05 by two-way ANOVA with Tukey's post hoc test). Tumor groups are distinguished by color and fill across the graph panels in A-D: Black (hollow): 4T1-Ctl + IgG, blue (hollow): 4T1-Ctl + α PD-L1, green (solid): 4T1-PD-L1 O/E + IgG, red (solid): 4T1-PD-L1 O/E + α PD-L1. (E) KEGG pathway analysis of DEGs upregulated in control tumors treated with α PD-L1 compared with IgG-treated controls. (F) KEGG pathway analysis of DEGs downregulated in PD-L1 overexpressing tumors treated with α PD-L1 compared with control tumors treated with α PD-L1. PD-L1, programmed death-ligand 1; α PD-L1, anti-PD-L1 antibody; CD45, cluster of differentiation 45; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; IgG, immunoglobulin G; O/E, overexpression.

Tumor cells adaptively increase PD-L1 levels to avoid immune attack via the PD-1/PD-L1 pathway, and this upregulation of PD-L1 inhibits the effect of ICIs (36). Upregulated

PD-L1 forms an immunosuppressive TME (37-39), which could reduce the effectiveness of ICIs. In the present study, in tumors overexpressing PD-L1, an increase in the total immune

cell levels was observed but a reduced infiltration of T cells involved in the anti-PD-L1 effect was also observed. In the control group with high anti-PD-L1 treatment efficacy, the Th17 cell differentiation KEGG pathway was highly expressed. Th17 recruits antitumor neutrophils and CD8⁺ cytotoxic T cells to promote tumorigenic mediators, which is consistent with the findings (40). Additionally, in the group that responded to treatment, the Th1/Th2 cell differentiation pathway was upregulated in KEGG pathway analysis. By contrast, this pathway was suppressed in PD-L1-overexpressing tumors that did not respond well to treatment. These findings are consistent with a previous study demonstrating that Th1 cells enhance antitumor immunity by secreting cytokines such as IFN- γ and IL-2, which promote the proliferation and cytotoxic activity of CD8⁺ T cells (41). Th1 activity also contributes to antitumor effects by promoting M1 macrophage polarization (42). By contrast, Th2 cells facilitate tumor growth by secreting immunosuppressive cytokines through M2 macrophages (43). Furthermore, in the present study, NK cell-mediated cytotoxicity pathways were downregulated in the PD-L1-overexpressing group. As critical effectors of innate immunity, NK cells contribute to antitumor responses by promoting dendritic cell maturation and inducing IL-12p70 production, which further supports Th1 polarization (44). These findings suggest that the reduced efficacy of anti-PD-L1 therapy in PD-L1-upregulated tumors may be attributed to impaired Th1 responses and attenuated NK cell activity, both of which are essential for effective antitumor immunity. The observed immunosuppressive phenotype in PD-L1-overexpressing tumors aligns with the well-established role of the PD-1/PD-L1 axis in dampening T cell activation and promoting the expansion of M2 macrophages (45,46). These observations, derived from KEGG pathway analysis, support a model in which excessive PD-L1 expression contributes to a shift away from Th1- and NK cell-mediated immunity, potentially limiting the effectiveness of immune checkpoint blockade. These results indicate that PD-L1 overexpression may create an immunosuppressive state within the tumor immune microenvironment, thereby inhibiting the therapeutic efficacy of ICIs.

Similar observations have been reported across various cancer types regarding the role of the PD-1/PD-L1 pathway. In lung cancer, PD-L1 expression is associated with the suppression of CD8⁺ T cell activation and poor survival outcomes, reflecting the immunosuppressive role of the PD-1/PD-L1 axis in modulating antitumor immune responses (47). Furthermore, in colorectal cancer, PD-L1 expression contributes to immune evasion by enhancing regulatory T cell function and inducing IL-10 expression (48). PD-L1 expression is more frequently observed in metastatic tumors, where it correlates with poor prognosis and serves as a biomarker for immunotherapy eligibility (49). In gastric cancer, the expression of PD-1 and PD-L1 in tumor-infiltrating immune cells has been associated with a favorable prognosis (50). By contrast, tumor-associated fibroblasts promote PD-L1 expression in cancer cells, thereby contributing to an immunosuppressive microenvironment and facilitating tumor progression (51). In pancreatic cancer, PD-L1-positive tumors have been shown to exhibit increased infiltration of regulatory T cells, reduced levels of IL-10 and increased interferon- γ production following PD-L1 blockade,

further supporting the therapeutic potential of targeting the PD-1/PD-L1 axis (52).

Combination therapy with neoadjuvant chemotherapy and ICIs has been used to improve tumor response and survival outcomes in TNBC (13,34). Surgical excision is important in the treatment of TNBC, and in cases that involve non-palpable breast cancer, minimal localization procedures would make treatment precise and minimize complications (53-55). The interplay between immune-based therapies and evolving surgical techniques might provide a more extensive approach to TNBC treatment. However, the molecular function of PD-L1 upregulation in TNBC tumors was not elucidated in the present study and the clinical significance of PD-L1 in human tumor tissue samples was not demonstrated, both of which are the major limitations of the present study.

In conclusion, the present study showed that the efficacy of anti-PD-L1 in immunotherapy for TNBC is associated with the PD-L1 expression status of the tumor. PD-L1 upregulation induces changes in the tumor immune environment, which may be the main cause of the limitations of immunotherapy. These results indicate that novel therapeutic strategies considering the role of PD-L1 should be further explored.

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Availability of data and materials

The RNA sequencing data generated in the present study may be found in the NCBI Gene Expression Omnibus repository under accession number GSE292013 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE292013>. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

AYP and JHK confirm the authenticity of all the raw data. Conception, design and methodology development were conducted by JHK, HSK, HKK, HBL and WH; acquisition of data (including experimentation and data collection) was conducted by JHK, AYP, SL, HKK and HBL; analysis and

interpretation of data (including statistical and bioinformatics analysis) was conducted by JHK, AYP, HKK, HBL and WH; writing and/or revising the manuscript was conducted by JHK and WH; administrative, technical or material support (including facility use and technical assistance) was provided by HKK and HBL; study supervision and project administration were conducted by WH. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

In vivo experiments were conducted at the animal facility of Seoul National University Hospital (Seoul, Republic of Korea), following institutional guidelines and obtaining prior approval from the Institutional Animal Care and Use Committee (approval no. 23-0157-S1A0). Animals were housed in an AAALAC International-accredited facility (accreditation no. 001169) in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition, NRC (2010).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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