Metformin Suppresses Both PD-L1 Expression in Cancer Cells and Cancer-Induced PD-1 Expression in Immune Cells to Promote Antitumor Immunity

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SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

Rabbit polyclonal antibodies that recognize human β-catenin (pS552; #9566; 1:1,000 for immunoblotting), β-catenin (#8814; 1:1,000 for immunoblotting), human PD-L1 (E1L3N; #13684; 1:1,000 for immunoblotting), AKT (pS473; #4060; 1:2,000 for immunoblotting), AKT (#9272; 1:1,000 for immunoblotting), PD-1 (#86163; 1:1,000 for immunoblotting and 1:100 for immunoprecipitation), mouse PD-1 (#84651; 1:1,000 for immunoblotting), AMPKα (pT172; #2535; 1:1,000 for immunoblotting), AMPKα (#5831, 1:1,000 for immunoblotting), and V5-Tag (#13202; 1:2,000 for immunoblotting and 1:100 for immunoprecipitation) were purchased from Cell Signaling Technology (Danvers, MA, USA). Actinomycin D (#A1410), cycloheximide (CHX) (#66-81-9), MG132 (#1211877-36-9), metformin (#1115-70-4), compound C (#866405-64-3), and mouse monoclonal antibodies against FLAG (#F3165, clone M2; 1:5,000 for immunoblotting) and tubulin (#T6074; 1:5,000 for immunoblotting) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hygromycin (#400053) was purchased from EMD Biosciences (San Diego, CA, USA).

Cell culture

A375SM human melanoma cells (#80004), A431 human epidermoid carcinoma cells (#21555), U87MG glioblastoma (GBM) cells (#30014), MDA-MB-231 human breast carcinoma cells (#30026), and B16F10 murine melanoma cells (#80008) were purchased from the Korean Cell Line Bank (Seoul, Korea). HEK293T cells (#CRL-3216) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). U251 GBM cells were kindly provided by Dr. In Ah Kim (Seoul National University, Seoul, Korea). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM;

Welgene, Gyeongsan-si, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene) and 1% penicillin/streptomycin (Capricorn, Düsseldorf, Germany). AMPK wild-type (WT) and AMPKα1/2 double-knockout (AMPK DKO) mouse embryonic fibroblasts (MEFs) were kindly provided by Dr. Benoit Viollet (Université Paris Descartes, Paris, France) and maintained in doxycycline-containing DMEM. Jurkat cells (#TIB-152; ATCC) and C57BL/6 mouse primary splenocytes were maintained in RPMI 1640 (HyClone, Logan, UT, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. NK-92 natural killer (NK) cells (#CRL-2407; ATCC) were maintained in X-VIVO (Lonza, Walkersville, MD, USA). Human primary NK cells were maintained in CTS AIM V (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with human platelet lysate (Sigma-Aldrich) and 1% penicillin/streptomycin. All cells were routinely tested for mycoplasma, with negative results.

Transfection and DNA constructs

Cells were seeded at a density of 4×10^5 cells in a 60-mm dish or 1×10^5 cells/well in a 6-well plate 18 hrs before transfection. Transfection was performed using Lipofectamine 2000 Transfection Reagent (#11668019; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transfected cells were stabilized for 24 hrs and selected by incubation with 100 μg/mL hygromycin for one week. pLV/β-catenin deltaN90 (CA-β-catenin; #36985), pECE-Myr-HA-AKT1 (delta4-129; #10841), and pAAV-EF1a-Flex-CA-AMPK–H150R-T2A-mCherry (CA-AMPK; #83490) were purchased from Addgene (Cambridge, MA, USA). Human PD-1 was cloned into pcDNA6/V5-His. The primers used for expression plasmid construction were as follows: human *Pdcd1*, 5′-AATAGAATTCATGCAGATCCCACAGGCGCCCT-3′ (forward) and 5′-ATATGATATCGAGGGGCCAAGAGCAGTGTC-3′ (reverse).

Quantitative real-time PCR analysis

Total RNA was isolated using TRIsure reagent (#BIO-38033; Bioline, London, UK) according to the manufacturer's instructions. Equal amounts of total RNA were used for cDNA synthesis using PrimeScript RT Master Mix (Perfect Real Time) (#RR036A; Takara Bio, Kyoto, Japan). Real-time PCR was performed on an ABI Prism 7500 system using SYBR Green PCR Master Mix (#43-091-55; Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The PCR conditions were as follows: 40 cycles of denaturation at 95°C for 15 secs and amplification at 60°C for 1 min. Relative expression levels were calculated using the comparative cycle threshold method [1] and normalized to that of the housekeeping gene *Hprt1*. All reactions were run in triplicate. The following primer pairs were used: human *CD274*, 5′-CTGCACTTTTAGGAGATTAGATC-3′ (forward) and 5′-CTACACCAAGGCATAATAAGATG-3′ (reverse); human *Pdcd1*, 5′-GACAGCGGCACCTACCTCTGTG-3′ (forward) and 5′-GACCCA GACTAGCAGCACCAGG-3′ (reverse); mouse *Pdcd1*, 5′-CGGTTTCAAGGCATGGTCATTGG-3′ (forward) and 5′-TCAGAGTGTCGTCCTTGCTTCC-3′ (reverse); *Hprt1*, 5′-CATTATGCTGAGGATTTGGAAAGG-3′ (forward) and 5′-CTTGAGCACACAGAGGGCTACA-3′ (reverse).

Immunoprecipitation and immunoblotting analysis

Proteins were extracted from cultured cells using a modified buffer followed by immunoprecipitation and immunoblotting with corresponding antibodies as described previously [2]. Proteins were extracted from cultured cells using a lysis buffer (50 mM Tris-HCl [pH 7.5], 0.1% sodium dodecyl sulfate, 1% Triton X-100, 150 mM NaCl, 1 mM

dithiothreitol, 0.5 mM EDTA, 100 μM sodium orthovanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, and proteinase inhibitor cocktail) and centrifugation at 12,000 × *g*, 4°C for 15 mins. Protein concentrations were determined using a DC protein assay kit (#5000112; Bio-Rad, Hercules, CA, USA). Equal amounts of lysates were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk at room temperature (25°C) for 30 mins and then incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, #NA934V or anti-mouse IgG, #NA931V; Sigma-Aldrich) at room temperature for 2 hrs.

For immunoprecipitation, cultured cells were lysed using an immunoprecipitation buffer to extract proteins. Equal amounts of lysates were incubated with primary antibodies at 4°C overnight. Then, protein A resin (#L00210, GenScript, Piscataway, NJ, USA) was added, followed by incubation at 4°C for 3 hrs. Immunocomplexes were washed with an immunoprecipitation buffer three times and then subjected to immunoblot analyses with the indicated antibodies. Band intensities were quantified using the ImageJ 1.53e software (National Institutes of Health, Bethesda, MA, USA). Each experiment was repeated at least three times.

Ubiquitylation assay

The ubiquitylation assay was performed as previously described [3]. Briefly, cells cultured under appropriate conditions or transfected with indicated plasmids were lysed using a denaturation buffer (6 M guanidine-HCl [pH 8.0], 0.1 M Na_2HPO_4/NaH_2PO_4, and 10 mM imidazole) containing 5 mM N-ethylmaleimide to prevent deubiquitylation. The cell lysates

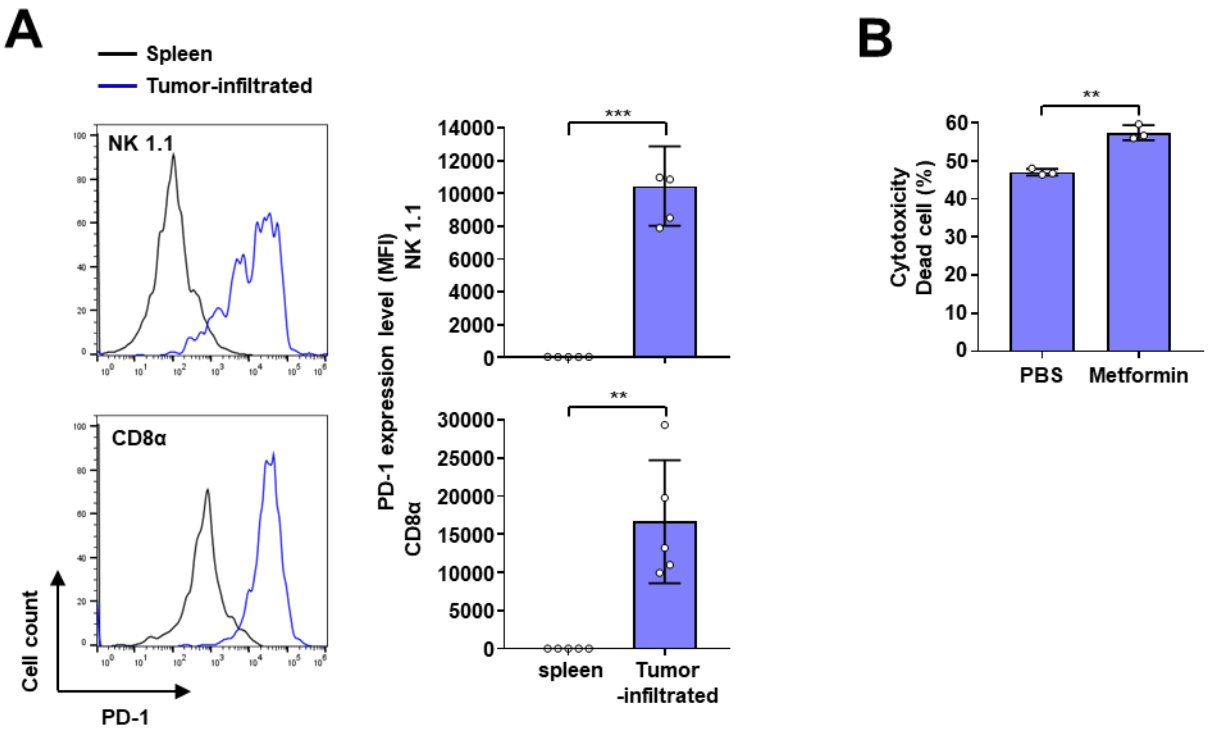
were immunoprecipitated using the indicated antibodies, washed, and subjected to immunoblotting analysis using the indicated antibodies.

Preparation of conditioned medium (CM)

A431 and B16F10 cells were cultured in DMEM complete medium in 10-cm tissue culture dishes until confluence (70%). The cells were washed three times with serum-free DMEM and cultured in complete media based on fresh RPMI 1640 (for splenocytes and Jurkat cells), CTS AIM V (for human primary NK cells), or X-VIVO (for NK-92 cells). After 24 hrs of incubation, CM was harvested, centrifuged at 2,000 rpm for 5 mins, and stored at –80°C.

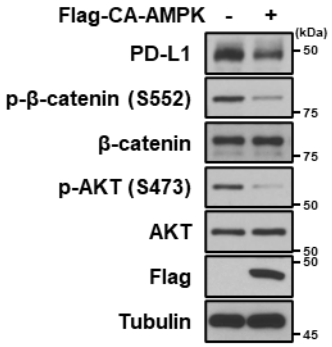
Flow-cytometric analysis

Harvested tumor tissues were dissociated to obtain single-cell suspensions using a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and a Tumor Dissociation Kit (#130-096-730; Miltenyi Biotec) according to the manufacturer's instructions. The single-cell suspensions were washed with phosphate-buffered saline (PBS) and stained with the indicated antibodies (anti-mouse PD-1 antibody, #135213; anti-mouse PD-L1 antibody, #124313; anti-mouse CD45 antibody, #103105; anti-mouse CD8a antibody, #100721; and anti-mouse NK-1.1 antibody, #108707; BioLegend, San Diego, CA, USA) in a flow cytometry blocking buffer (0.5% BSA and 2% FBS in PBS) at 4°C for 30 mins. After staining, the cells were washed three times with flow cytometry buffer (0.5% BSA in PBS) and measured using a BD FACSCelesta flow cytometer (BD Biosciences, San Diego, CA, USA).



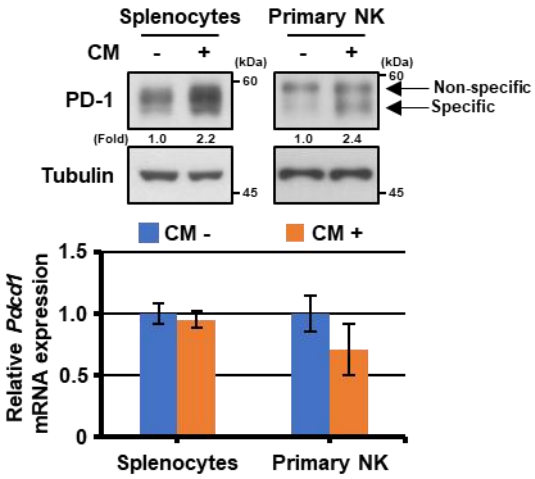
Supplemental Data Fig. S1. (Related to Fig. 1). Metformin inhibits both PD-L1 expression in cancer cells and PD-1 expression in infiltrating immune cells in the TME. (A) Cell-surface analysis of PD-1 protein expression in NK cells or $CD8^+$ T single-cell suspensions derived from tumors and splenocytes was performed using a flow cytometer. A representative histogram (left panel) and graph (right panel) are shown. (B) Human primary NK cells and A431 cells were pretreated with PBS and metformin (10 mM) for 24 hrs, respectively, and then cocultured for 4 hrs. CFSE fluorescence and the viability status indicated by Fixable Viability Dye were quantified using flow cytometry. Data are presented as mean ± SD of three independent experiments. $^{**}P < 0.01$, Student's *t*-test.

Abbreviations: PD-L1, programmed death ligand 1; PD-1, programmed death 1; TME, tumor microenvironment; NK, natural killer; PBS, phosphate-buffered saline; CFSE, carboxyfluorescein succinimidyl ester.



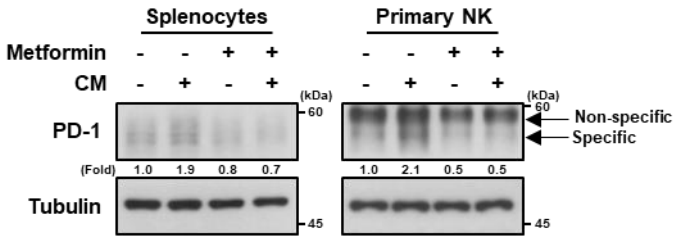
Supplemental Data Fig. S2. (Related to Fig. 2). AMPK inhibits PD-L1 expression. A431 cells were stably transfected with Flag-vector or Flag-CA-AMPK. Immunoblotting analyses were performed using the indicated antibodies.

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; PD-L1, programmed death ligand 1; CA, constitutively active.



Supplemental Data Fig. S3. (Related to Fig. 3). Cancer cells enhance PD-1 protein levels but not mRNA levels in immune cells. C57BL/6 mouse primary splenocytes and human primary NK cells were treated or not with CM (50%) for 12 hrs. Immunoblotting (upper panel) and quantitative real-time PCR (bottom panel) were performed using the indicated antibodies and primers, respectively. Data are presented as mean ± SD of three independent experiments.

Abbreviations: PD-1, programmed death 1; NK, natural killer; CM, conditioned medium.



Supplemental Data Fig. S4. (Related to Fig. 4). Metformin inhibits CM-induced PD-1 upregulation in immune cells. C57BL/6 mouse primary splenocytes and human primary NK cells were treated or not with CM (50%) or metformin (10 mM) for 12 hrs. Immunoblotting analyses were performed using the indicated antibodies.

Abbreviations: CM, conditioned medium; PD-1, programmed death 1; NK, natural killer.

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3. Lee JH, Liu R, Li J, Zhang C, Wang Y, Cai Q, et al: Stabilization of phosphofructokinase 1 platelet isoform by AKT promotes tumorigenesis. Nat Commun 2017;8:949.