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Pomalidomide Inhibits PD-L1 Induction to Promote Antitumor Immunity

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Abstract

Thalidomide-like drugs have been approved for the treatment of human multiple myeloma, with their direct antitumor effects and immunomodulatory functions well documented. However, the exact molecular mechanisms that govern these effects remain unclear. Here we demonstrate that pomalidomide promotes immune response by inhibiting expression of PD-L1. Pomalidomide inhibited PD-L1 expression on tumor cells to promote CTL activity *in vitro* and suppressed PD-L1 upregulation on antigen-presenting cells to prevent peptide-induced T-cell tolerance. Knockout of PD-L1 on tumor cells or in mice completely eliminated the immunomodulatory effect of pomalidomide. Furthermore, pomalidomide synergized with other immunotherapies to improve anticancer therapy. Taken together, this study identifies a new mechanism for the immunomodulatory functions of pomalidomide in cancer therapy. These results also offer a clinical approach for blocking PD-L1 induction and potentially promoting antitumor immunity.

Significance: These findings report that the immunomodulatory drug pomalidomide, widely used to treat myeloma and other cancers, enhances antitumor immunity by inhibiting PD-1/PD-L1 expression. *Cancer Res; 78(23); 6655–65. ©2018 AACR.*

Introduction

The PD-1 and PD-L1 pathway plays a key role in cancer immune escape and the establishment of a suppressive tumor microenvironment (TME; ref. 1). PD-1 is upregulated on activated T cells upon T-cell receptor (TCR) engagement and is highly expressed on exhausted T cells in chronic infection and tumor-infiltrating lymphocytes (TIL) in cancer (2, 3). Its major ligand PD-L1 (B7-H1) is normally expressed on tissue macrophages but can be rapidly induced on the majority of tissue cells by inflammatory cytokines in a variety of disease conditions (4, 5). PD-1 interacts with another ligand PD-L2 (B7-DC), which has restricted expression on dendritic cells (DC) and a subset of B cells (6). When PD-1 on activated T cells encounters its ligands, T-cell effector functions are inhibited, and ultimately T cells become tolerant, dysfunctional, or exhausted (7). Cancer cells utilize this mechanism to evade immune attach by upregulate PD-L1 expression. Antibodies targeting both PD-1 and PD-L1 are able to revive endogenous antitumor immune responses and have been approved for clinical usage in multiple cancer types (8, 9).

PD-L1 expression is associated with prognosis in several cancer types and is arguably the most reliable predictor for oncologic response to PD-1/PD-L1 inhibitor (10). Surface PD-L1 is found on both tumor cells and immune cells within the TME, and both cellular compartments contribute to PD-L1–mediated immune suppression in a context-dependent perspective (11). PD-L1 overexpression on tumor cells could be caused by intrinsic signaling changes or induced by the TME. Alterations in oncogene signaling pathways have been identified to regulate PD-L1 transcription, including PTEN, EGFR, KRAS, MYC, and ALK, and many of them affect constitutive PD-L1 expression on tumor cells (12–14). The induction of PD-L1 within the tumor is mainly regulated by inflammatory cytokines. Among them, IFNγ secreted by TILs is the major inducer for PD-L1 upregulation at tumor sites, a phenomenon called adaptive immune resistance (1, 10). Consistent with this observation, patients with cancer with defects in the IFNγ receptor pathway are unresponsive to PD-1 therapy in the clinic (15, 16).

Thalidomide-like drugs, including thalidomide, lenalidomide, and pomalidomide, are a group of immunomodulatory drugs (IMiD), and they have contributed to the survival improvement in patients with relapsed or refractory multiple myeloma (17, 18). Besides their direct tumoricidal effect and inhibition of angiogenesis and inflammation, IMiDs possess immunomodulatory functions, including costimulating T cells, Th1-type skewing, as well as enhancing natural killer (NK) and NKT cells activities (19, 20). Many of these immunomodulatory functions are presumably mediated through the protease cereblon (CRBN) for the degradation of its downstream targets (21). The *in vivo* immunomodulatory effects of IMiDs have also been reported in several mouse solid cancer models (22, 23), although mouse CRBN is known to not interact with thalidomide-like agents, suggesting a

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CRBN-independent immunomodulatory effect (24, 25). Thus, the exact molecular mechanism, as well as the relative importance of the immunomodulatory effects of thalidomide-like drugs in cancer therapy, is yet to be dissected.

Here we investigated the molecular mechanism of the immunomodulatory effects of thalidomide-like drugs, mainly pomalidomide, in the antitumor response. We found that pomalidomide inhibits the expression of inducible PD-L1 to promote T-cell response, as well as T-cell–mediated antitumor immunity.

Materials and Methods

Chemicals and antibodies

Pomalidomide, purchased from Selleck Chemicals, was dissolved in DMSO and stored at −80°C until use. Thalidomide and lenalidomide were purchased from Sigma-Aldrich. Mouse or human recombinant IFNγ were purchased from BioLegend and stored at −80°C until use. Albumin from chicken egg white and polyinosinic-polycytidylic (Poly I:C) were purchased from Sigma-Aldrich. OVA peptide (SIINFEKL) was synthesized by GenScript. Anti-41BB (CD137) mAb (clone; 3H3), anti-mouse CD8β mAb (clone; 53-5.8), anti-mouse CD4 mAb (clone; GK1.5), and anti-mouse PD-1 (CD279) mAb (clone; RMP1-14) were purchased from BioXcell. Antibodies for flow cytometry staining, if not specified, were purchased from BioLegend. Hybridoma for mouse PD-L1 neutralizing mAb (clone 10B5) was obtained from Dr. Lieping Chen's laboratory at Yale University (New Haven, CT; ref. 26).

Cell lines

MC38 (colon adenocarcinoma), B16-OVA (melanoma), SK-MEL-624 (melanoma), and SKOV-3 (ovarian carcinoma) were acquired from Dr. Lieping Chen (Yale University, New Haven, CT). Pancreatic adenocarcinoma cells Pan02 and BxPC-3 were from Dr. Colin Weekes (University of Colorado AMC, Aurora, CO). Multiple myeloma 8226 and 5TGM1 were from Dr. Fenghuang Zhan (University of Iowa, Iowa City, IA). Tumor cells were cultured in DMEM or RPMI1640 containing 10% FBS and 1% penicillin/streptomycin. KPC cell, which was derived from $KrasLSL^{G12D}/+$; $Trp53^{R172H}$; Pdx1-Cre (KPC) transgenic mice, was obtained from Dr. Lei Zheng's laboratory at the Johns Hopkins University (Baltimore, MD; ref. 27). *PD-L1* and *CRBN* genes in tumor cells were knocked out by the CRISPER/Cas9 technology (28). Briefly, tumor cells were temporary transfected with a Cas9-single guide RNA (sgRNA) expression vector (pX459; Addgene) targeting PD-L1 (sgRNA, 5′-GGTCCAGCTCCCGTTC-TACA-3′) or CRBN (sgRNA, 5′-GTCCTGCTGATCTCCTTCGC -3′). Cells were incubated with puromycin (MC38: 4 μg/mL; B16-OVA, SK-MEL-624, and SKOV-3: 1 μg/mL) for 7 days to enrich puromycin-resistant cells. After stimulation with mouse recombinant IFNγ (10 ng/mL) for 24 hours, $PD\text{-}L1^{KO}$ cells were isolated by sorting out PD-L1–negative cells. $CRBN^{KO}$ cells were seeded by limited dilution and then selected by Western blot analysis. MC38 cells constitutively expressing high PD-L1 ($MC38^{PD\text{-}L1}$) were generated by transducing $MC38^{PD\text{-}L1\ KO}$ cells with a mouse PD-L1 construct, and then selected via flow cytometry sorting. All cell lines in the laboratory were tested for *Mycoplasma* infection by DAPI staining upon purchase or obtaining, and have been kept as stocks in liquid nitrogen tank before growing for use up to three weeks in culture. Morphology and growth curve analysis were performed regularly, and no phenotypic changes were observed throughout the study.

Mice

Wild-type C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory. OT-1 transgenic mice were kindly provided by Dr. Ross M. Kedl (Department of Immunology and Microbiology, University of Colorado AMC, Aurora, CO). $PD\text{-}L1^{-/-}$ mice in C57BL/6 background (29) were kindly gifted from Dr. Raphael A. Nemenoff (Department of Renal Disease and Hypertension, University of Colorado AMC, Aurora, CO). All animal care, experiments, and euthanasia were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus (Aurora, CO).

Flow cytometry

Single-cell suspensions were blocked with LEAF anti-mouse CD16/32 (anti-FcγIII/II receptor, clone 93) for 20 minutes before staining with primary conjugated antibodies. Samples were analyzed by a BD FACSCalibur or a BD LSRFortessa cell analyzer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

RT-PCR and qRT-PCR

Samples were incubated with or without pomalidomide as well as recombinant IFNγ for 5 hours. RNA was extracted using RNAqueous Micro Kit from Invitrogen, and cDNA was synthesized using PrimeScript RT-PCR Kit (Takara). The primer sequences for amplification were as follows; 5′-ATGGTGGTGCC-GACTACAA-3′ (forward), 5′-TCCAGATGACTTCGGCCTT-3′ (reverse) for PD-L1; and 5′-GGGAGCGAGATCCCTCCAAAAT-3′ (forward), 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative PCR was performed using by TaqMan Gene Expression Assay (Applied Biosystems).

Transcriptome analysis

Total RNA was extracted using RNAqueous Micro Kit (Invitrogen) as per the manufacturer's instructions. RNA library preparation and sequencing were conducted by the Genomics and Microarray Core at University of Colorado Cancer Center (Aurora, CO). The 151-bp single-end fastq files generated from Illumina NextSeq500 platform were aligned to the UCSC human hg19 reference genome using the STAR 2.4.0 RNA-seq aligner. The aligned reads were processed by featureCounts software to understand strand information. Gene quantification to hg19 reference gene annotation was performed using Cuffquant module of Cufflinks package. Gene ontology analysis was performed using the ToppGene web tool (https://toppgene.cchmc.org/). Protein association network was constructed using the STRING web tool (https://string-db.org/). Differential expression test between two sample groups was performed using Cuffdiff module. Genes with P_{adj} values <0.05 between two groups were chosen as differentially expressed genes. The data have been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE120844 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE120844).

Western blot analysis

Protein was extracted from $CRBN^{WT}$ or $CRBN^{KO}$ cancer cells by protein extraction reagent (Thermo Fisher Scientific), and Western blot analysis was performed. All primary and secondary antibodies were purchased from Cell Signaling Technology. After

incubating with primary antibody overnight, membranes were incubated with the peroxidase-labeled secondary antibody for 2 hours and detected by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Images were obtained and quantified via Chemi Doc XRS+ and Image Lab Software (Bio-Rad).

In vitro coculture between tumor cells and CTL

We stimulated OT-1 T cells with SIINFEKL peptide over 7–10 days and subsequently used as CTLs to culture with B16-OVA. For MC38 cells, we generated alloreactive CTLs by stimulating BALB/c T cells with fluorouracil (5-FU)-treated black B6 splenocytes over 7 days. Tumor cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) before culturing with activated CTLs for 24 hours at a ratio of 1:2 or 1:4 (tumor cells: lymphocytes) in 24-well plate. Purified anti-mouse PD-1 antibody (10 μg/mL) as well as pomalidomide (10 μmol/L) were added if necessary. After incubation, the expression of PD-L1 and cell death, measured by propidium iodide (PI) staining, were analyzed by FACS. Supernatants were collected for the measurement of IFNγ concentration by ELISA (Beckton Dickinson).

Mouse tumor model

MC38 (5×10^5) or B16-OVA (2×10^5) cells were subcutaneously injected into the right flank of B6 mice. Mice were randomized to different treatment groups. Pomalidomide (50 mg/kg) was injected intraperitoneally to mice twice a week. Tumor diameter (length × width) was measured every 2 or 3 days with a caliper. Tumor volume was determined as $1/2 \times (\text{length} \times \text{width}^2)$. Mice were euthanized and sacrificed if the length of tumor reached 20 mm or a tumor became ulcerated.

Mouse OT-1 T-cell anergy model

T cells were taken from lymph nodes and spleens of CD45.1$^+$ OT-1 TCR transgenic mice and then OT-1 T-cells were isolated by mouse Pan T-cell Isolation Kit (Miltenyi Biotec). Purified OT-1 T cells (1×10^6) were intravenously injected to C57BL/6 or PD-L1$^{-/-}$ mice. On the same day, OVA peptide (0.5 mg) was intravenously injected to these mice. Pomalidomide (50 mg/kg) was intraperitoneally injected both 24 and 72 hours after OVA peptide injection.

Single-cell isolation from fresh tumor tissues

Mouse tumor tissues were collected, cut into small pieces, and resuspended in RPMI1640 medium with Liberase (Roche Diagnostics Corporation). Tumors were digested for 50 minutes in a shaking incubator at 37°C. After removing Liberase by centrifugation, digested tissues were passed through a 100-μm cell strainer to make single-cell suspensions. Cells were incubated with 2 mL of ACK lysis buffer at room temperature for 2 minutes. White blood cells were separated from tumor cells using Percoll (Sigma-Aldrich) density gradients. White blood cells were collected between the lower layer (60% Percoll) and middle layer (30% Percoll) by centrifugation at 1,000 × *g* for 30 minutes.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics 20 version software for Mac. All figures were generated using GraphPad Prism 7.0 software (GraphPad Software). The Mann–Whitney *U* test was performed to compare differences in the nonparametric variables between the groups. The log-rank test was performed to compare the survival and also the repeated measures ANOVA was performed to compare time-dependent tumor growth. Each sample *in vitro* was analyzed in triplicate samples. All *P* values less than 0.05 were considered to indicate significance.

Results

Pomalidomide triggers T-cell–dependent antitumor immunity

To assess the immunomodulatory role of IMiDs on the antitumor response *in vivo*, we tested the *in vivo* antitumor effect of pomalidomide on MC38, an immunogenic mouse colon cancer cell line. Immunocompetent wild-type C57BL/6 mice were subcutaneously implanted with MC38 tumor cells. One day after tumor implantation, mice were treated with intraperitoneal injection of pomalidomide twice a week for 2 weeks. As shown in Fig. 1A, multiple doses of pomalidomide treatment right after tumor inoculation significantly retarded tumor growth, and as a result, the pomalidomide-treated group has significantly better survival over the control group (Fig. 1B). We further evaluated the involvement of adaptive immunity for pomalidomide-mediated tumor regression, using depleting antibodies. Depletion of either CD4$^+$ or CD8$^+$ T cells (Fig. 1C) completely eliminated the antitumor effect promoted by pomalidomide, suggesting that the pomalidomide-triggered antitumor effect relies on both T-cell subsets.

We dissected tumor tissues to further determine the possible mechanisms through which pomalidomide promotes antitumor immunity. Tumor tissues were collected two weeks after tumor inoculation, when the pomalidomide-treated group showed clearly smaller tumor weight (Fig. 1D). We observed more T lymphocyte infiltrates (CD3$^+$ cells), accompanied with an increase in the ratio of CD8$^+$ T to CD4$^+$ T cells in tumors of the pomalidomide-treated group (Fig. 1E). We focused on examining the expression of B7 molecules at tumor sites, as they are known to be key regulators for T-cell immunity (3, 30). The expression of PD-L1 on antigen-presenting cells (APC) within tumors, including both MHC class II$^+$ and class II- CD11b$^+$ myeloid cells, was dramatically reduced by pomalidomide treatment (Fig. 1F and G). On the contrary, pomalidomide treatment only slightly affected the expression of other known B7 ligands, including B7-1, B7-2, and ICOSL, in both myeloid cell subsets (Supplementary Fig. S1A–S1F). Implanted MC38 tumor cells induced PD-L1 expression, presumably in response to massive lymphocyte infiltration, and pomalidomide treatment eliminated this upregulation (Fig. 1H). Therefore, our results suggest that pomalidomide treatment promotes CTL-mediated antitumor immunity through inhibiting PD-L1 expression on both tumor cells and nontumor cells within the TME.

Pomalidomide inhibits PD-L1 upregulation and promotes tumor killing by CTL

The reduced PD-L1 expression in MC38 tumor tissues from pomalidomide-treated mice led us to test whether pomalidomide can directly inhibit PD-L1 induction. First, we examined whether pomalidomide can inhibit PD-L1 upregulation on cancer cells by IFNγ, a major cytokine known to regulate PD-L1 expression. We selected four human cancer cell lines with different origins, SK-MEL-624, SKOV-3, 8226, and BxPC-3 as well as five mouse cancer cell lines, Pan02, KPC, B16-OVA, 5TGM1, and MC38. All cancer cells upregulated PD-L1 expression in response to IFNγ treatment overnight, and the presence of pomalidomide suppressed PD-L1 upregulation. Thalidomide and lenalidomide, the two other IMiDs, were also capable of inhibiting IFNγ-induced PD-L1

expression, although in a relatively weaker fashion (Fig. 2A–C; Supplementary Fig. S2A and S2B). We noticed that pomalidomide treatment had little effect on constitutive PD-L1 expression in MC38 and B16-OVA cells (Supplementary Fig. S2C), which implies that pomalidomide inhibits inducible rather than constitutive PD-L1 expression. By both RT-PCR and quantitative PCR, we further verified that pomalidomide inhibited IFNγ-induced PD-L1 transcription in human cancer cells (Fig. 2D and E). To investigate genome-wide expression of IFNγ and pomalidomide, RNA sequencing was performed using SK-MEL-624 cancer cells treated with or without IFNγ as well as pomalidomide (Fig. 2F). Pomalidomide comprehensively inhibited IFNγ-targeted genes (Supplementary Fig. S3A–S3F), including the IFNγ-JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 axis (Fig. 2F), which is required to activate PD-L1 expression by IFNγ in cancer cells (11).

Because IMiDs are known to bind CRBN to execute an antitumor effect in human multiple myeloma (21), we evaluated whether CRBN was required for pomalidomide to inhibit PD-L1 activation in human cancer cell lines. We generated CRBN knockout cancer cell lines using the CRISPR/Cas9 technology (31), and knockout of CRBN was confirmed by Western blot analysis (Supplementary Fig. S4A). There was no significant difference in PD-L1 expression by pomalidomide treatment between $CRBN^{WT}$ and $CRBN^{KO}$ cancer cells (Supplementary Fig. S4B and S4C). Therefore, our data support that the effect of IMiDs on PD-L1 expression is independent of the CRBN pathway.

Cancer cells upregulate PD-L1 expression to protect themselves in response to CTL killing, a phenomenon called adaptive immune resistance (10). We made efforts to determine whether pomalidomide could inhibit the induction of PD-L1 on cancer cells in the presence of CTLs. We labeled two mouse pancreatic cancer cell lines (Pan02 and KPC) with CFSE before coculturing with alloreactive T cells. Inclusion of PD-1–blocking mAb promoted tumor killing and IFNγ secretion, demonstrating the involvement of the PD-1/PD-L1 pathway in this assay. PAN02 increased PD-L1 expression when cocultured with allogenic lymphocytes, and this upregulation was inhibited in the presence of pomalidomide (Fig. 3A). At the same time, pomalidomide promoted tumor death with the presence of activated allogenic lymphocytes (Fig. 3B). Consistent with that, pomalidomide treatment enhanced CTL activity against cancer cells, as demonstrated by more IFNγ secretion in the culture (Fig. 3C). We found a similar effect of pomalidomide in KPC tumor cells (Fig. 3D–F). Because pomalidomide at a dose of 10 μmol/L does not affect tumor death itself, our results supported that pomalidomide sensitized cancer cells to CTL killing by inhibiting PD-L1 expression.

Pomalidomide is known to be able to target T cells directly to modulate T-cell responses (32). To further verify that

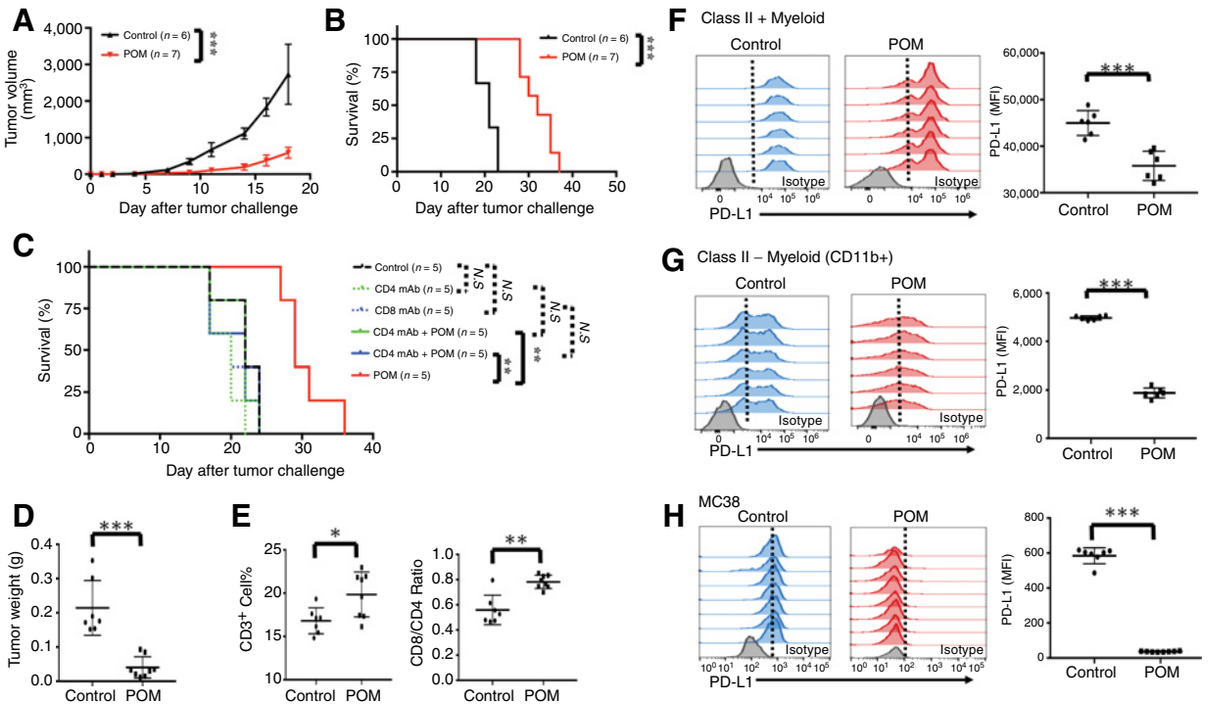

Figure 1.

Pomalidomide (POM) promotes T-cell–mediated antitumor immunity *in vivo*. Wild-type B6 mice were inoculated with MC38 tumors and were treated with pomalidomide or control. **A** and **B,** Median tumor volume (left) and tumor-related survival (right) were recorded once per two days. **C,** In some groups, mice were pretreated with depleting mAbs to remove respective cell types, and tumor-related survival was recorded. **D,** Tumor weight at 14 days after tumor inoculation is shown. **E,** In the tumor-infiltrating lymphocytes analysis, the percentages of infiltrating $CD3^+$ cells (T cells) and the ratio of CD8/CD4 cells are shown. PD-L1 expression in MHC class II^+ (**F**) and class II^- (**G**) myeloid cells within tumors (gated on $CD45^+CD11b^+$ cells), as well as MC38 tumor cells (**H**) isolated from tumor tissues. Data indicated mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *N.S.*, not significant).

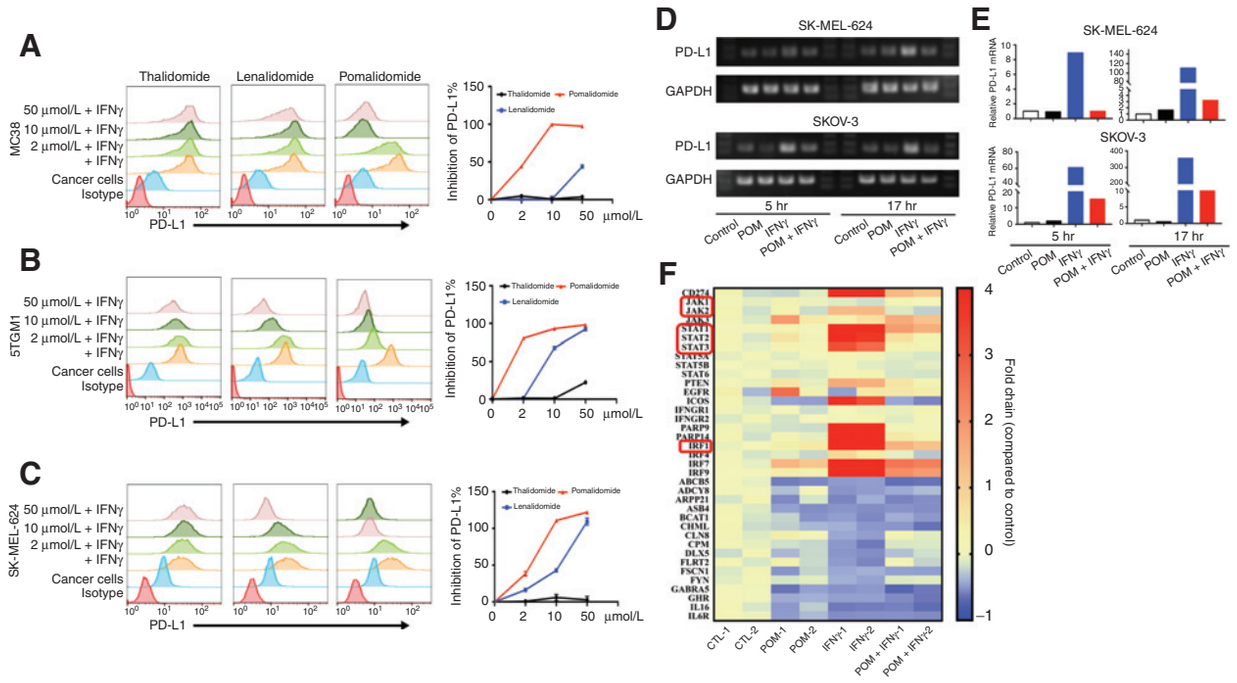

Figure 2.

IMiDs inhibit IFNγ-induced PD-L1 upregulation in cancer cells *in vitro*. Tumor cells were cultured with IFNγ alone, or together with different concentrations of IMiDs. Surface PD-L1 expression on MC38 (**A**), 5TGM1 (**B**), and SK-MEL-624 (**C**) was determined by flow cytometry. PD-L1 mRNA expression in SK-MEL-624 and SKOV-3 upon treating with IFNγ with or without pomalidomide (POM), was determined by PCR (**D**) or real-time PCR (**E**). RNA-seq of SK-MEL-624 cells pretreated with pomalidomide, IFNγ, or both for 10 hours. **F,** Heatmap of genes related to IFNGR signaling is indicated (fold change as compared with control samples).

pomalidomide acts on tumor cells, rather than T cells, to promote CTL activities, we preincubated Pan02 or KPC cells with pomalidomide for 1 hour, and then washed off pomalidomide before culturing tumor cells with CTL. In this setting, pomalidomide will not affect CTLs directly. As shown in Supplementary Fig. S5A–S5F, preincubation of tumor cells with pomalidomide at a higher dose (50 μmol/L) inhibited the induction of PD-L1 in both Pan02 and KPC cells. Although pomalidomide treatment itself did not kill tumor cells, the death of tumor cells pretreated with pomalidomide was significantly increased when cultured with activated alloreactive T cells (Supplementary Fig. S5B and S5E). Consistently, IFNγ secreted by T cells was also increased (Supplementary Fig. S5C and S5F). Thus, our results support the notion that pomalidomide acts on tumor cells to promote CTL activities indirectly.

PD-L1 is indispensable for pomalidomide to modulate T-cell–mediated antitumor immunity

Our data support that pomalidomide inhibits PD-L1 expression on cancer cells, so as to enhance CTL-mediated antitumor immunity. To directly test this, we generated PD-L1 knockout cancer cell lines using the CRISPR/Cas9 technology (31). PD-L1 knockout cell lines were screened and verified by the lack of surface PD-L1 upon IFN-γ stimulation. In contrast to MC38^{WT} cells (Fig. 4A–C) incubated with allogenic activated lymphocytes, pomalidomide treatment did not trigger any change in PD-L1 expression (Fig. 4D), tumor cell death (Fig. 4E), or IFNγ secretion (Fig. 4F) when PD-L1^{KO} MC38 cells were used. We observed similar results when PD-L1^{KO} B16-OVA cells were incubated with preactivated OT-1 T cells (Supplementary Fig. S6A–S6F). Altogether, our results demonstrated that pomalidomide inhibits inducible PD-L1 to promote CTL-mediated antitumor immunity.

Next, we inoculated the same numbers of WT and PD-L1^{KO} MC38 tumor cells onto B6 mice and then compared their response to pomalidomide treatment. PD-L1^{KO} MC38 cells grew slightly slower than wild-type MC38 tumor cells. Pomalidomide significantly retarded the growth of WT MC38 tumor cells, which is comparable to the effect of a commonly used mouse PD-L1 blocking mAb (Supplementary Fig. S7A and S7B; ref. 26). Importantly, pomalidomide treatment had no effect on tumor growth or survival in mice inoculated with PD-L1^{KO} MC38 tumors (Fig. 4G and H). We also compared tumor progression and survival between wild-type and PD-L1^{KO} B16-OVA tumor cells mediated by OVA vaccine with or without pomalidomide treatment. OVA vaccine had better antitumor effect in PD-L1^{KO} B16-OVA tumor than wild-type B16-OVA. In wild-type B16-OVA tumor, pomalidomide treatment significantly slowed tumor growth triggered by vaccine; in contrast, the addition of pomalidomide did not improve antitumor response or tumor-related survival by vaccine in PD-L1^{KO} B16-OVA tumors (Supplementary Fig. S7C and S7D). All these results support that PD-L1 on the tumor cell is required for pomalidomide-triggered antitumor immunity. On the other hand, we inoculated PD-L1$^{-/-}$ mice with MC38^{WT} tumors to investigate the involvement of host PD-L1. Tumor growth in PD-L1$^{-/-}$ mice was slightly slower than that in WT mice, suggesting a role for host PD-L1 in antitumor immunity. In PD-L1$^{-/-}$ mice, pomalidomide did not statistically inhibit tumor growth,

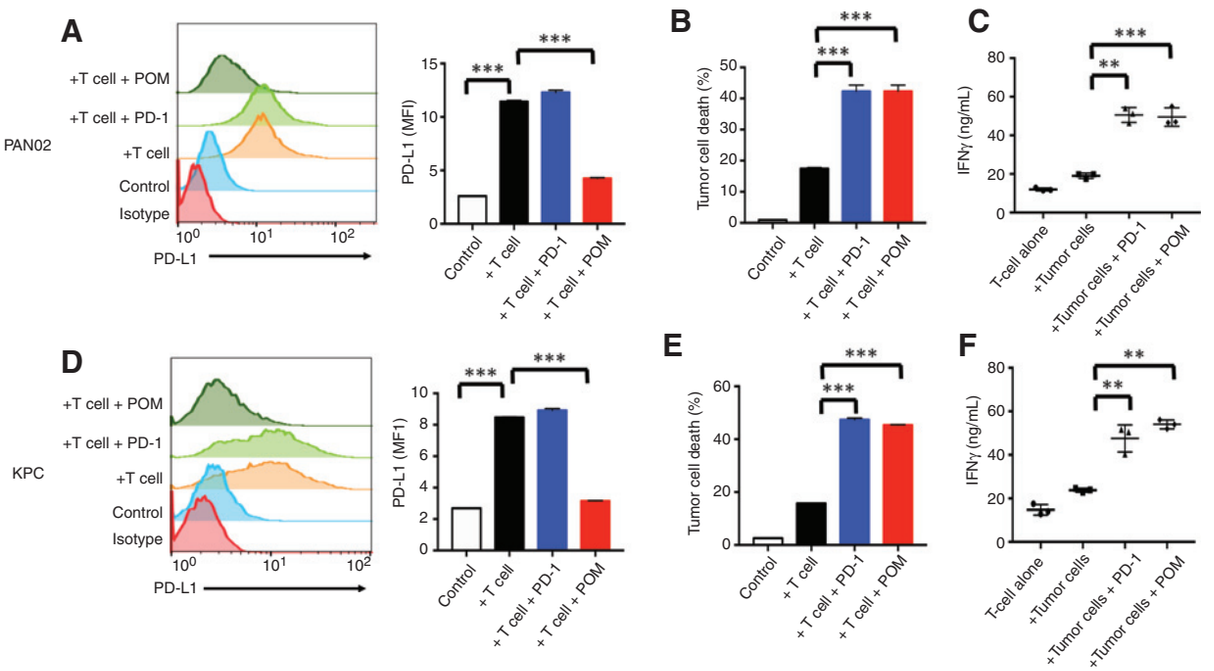

Figure 3.

Pomalidomide (POM) inhibits PD-L1 upregulation and promoted CTL activities against cancer cells *in vitro*. PD-L1 expression using mean fluorescent intensities (MFI) on Pan02 (**A**) or KPC (**D**) cells, which were cocultured with activated alloreactive T cells with or without pomalidomide (10 μmol/L) as well as PD-1 mAb (10 μg/mL) for 24 hours, is shown. Tumor cell death was determined by propidium iodide staining for Pan02 (**B**) or KPC (**E**) cells after coculture with alloreactive T cells. The concentration of IFNγ in the supernatant from Pan02 (**C**) or KPC (**F**) cells is shown. The ratio between tumor cells and alloreactive lymphocytes was 1 to 4. Data indicate mean ± SD. All samples are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *N.S.*, not significant).

but tumor size with pomalidomide treatment tended to be smaller (Supplementary Fig. S8A and S8B). This result suggests that PD-L1 of host cells plays a minor role in pomalidomide-triggered antitumor immunity.

Pomalidomide treatment prevents the induction of T-cell anergy via inhibiting PD-L1 expression

The PD-1/PD-L1 pathway is known to be required for antigen-induced T-cell tolerance (33, 34). We reasoned that by inhibiting PD-L1 expression, pomalidomide might be capable of preventing T cells from anergy induction *in vivo*. To test this, we used a peptide-induced T-cell anergy model (33). Indeed, transferred OT-1 T cells expanded dramatically in response to OVA peptide challenge, and there were significantly more OT-1 T cells in peripheral blood and spleens of the pomalidomide-treated group (Fig. 5A). PD-L1 is constitutively expressed on splenic APCs, including dendritic cells and macrophage/monocytes (1); OVA peptide inoculation was able to further increase PD-L1 expression, presumably because of the activation and expansion of transferred OT-1 T cells. Interestingly, pomalidomide treatment completely inhibited the upregulation of PD-L1 on splenic dendritic cells and macrophages (Fig. 5B). At the same time, there were consistently more OT-1 cells in peripheral blood of the pomalidomide-treated group. More importantly, when we rechallenged mice with OVA-peptide 21 days after first OVA-peptide challenge, only OT-1 T cells from pomalidomide-treated group were able to expand, but not those in control group (Fig. 5C). Thus, our study supports that pomalidomide treatment can prevent peptide-induced T-cell anergy.

To further test the requirement of PD-L1 in this model, we used PD-L1$^{-/-}$ mice as the host. When naïve OT-1 T cells were transferred into PD-L1$^{-/-}$ mice, peptide challenge dramatically increases the expansion of transferred OT-1 cells, which was consistent with published results (33). Importantly, pomalidomide treatment did not further increase the percentages of transferred OT-1 cells in PD-L1$^{-/-}$ mice (Fig. 5D). Taken together, our results support that pomalidomide inhibits PD-L1 induction to prevent peptide-induced T-cell tolerance.

Pomalidomide synergizes with other immunotherapies to treat established tumors

The immunomodulatory role of pomalidomide through inhibiting PD-L1 expression led us to further investigate whether the addition of pomalidomide could improve the anticancer efficacy of current immunotherapies. 4-1BB (CD137) is a classic costimulatory receptor for T cells, and amplifying its signal using a mAb has been demonstrated to be effective in many mouse tumor models, as well as in phase Ib clinical trials (35, 36). For delayed tumor treatment models, treatment began after 10 days when all tumors grew over 120 mm^3 (Fig. 6A). In the MC38 tumor model, delayed pomalidomide treatment alone had limited antitumor effect, although early intervention with pomalidomide could significantly retard tumor growth (Fig. 1A). In the MC38 tumor model, 4-1BB mAb treatment alone significantly slowed tumor

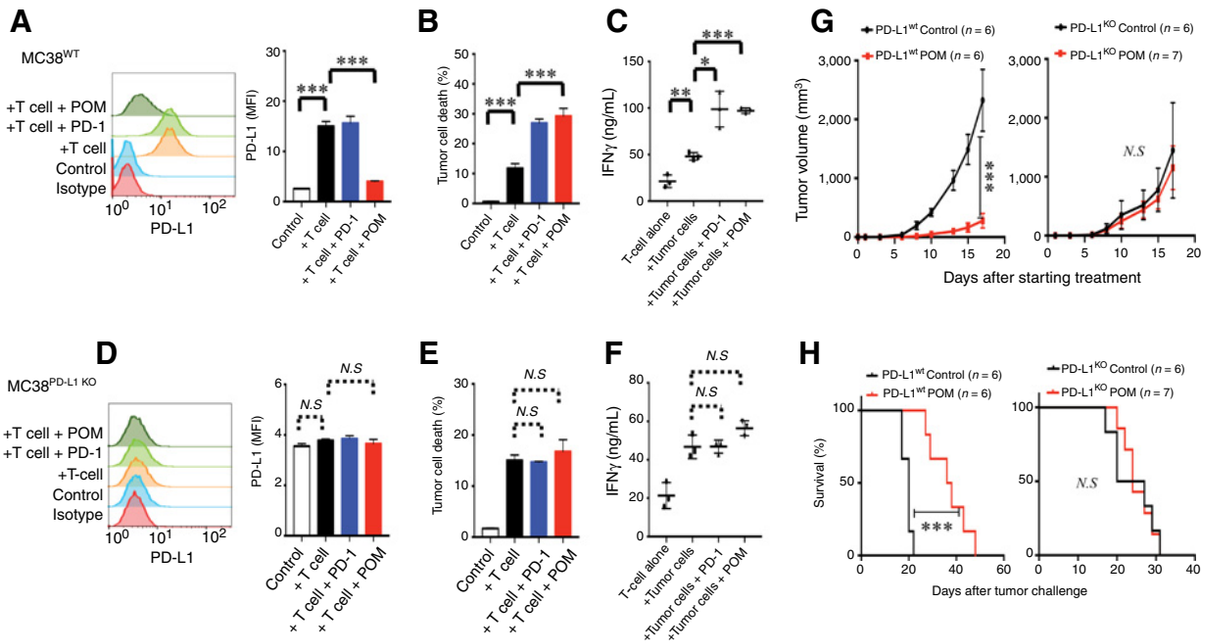

Figure 4.

PD-L1 is essential for pomalidomide (POM)-triggered antitumor T-cell immunity. MC38^{WT} (**A–C**) or MC38$^{PD\text{-}L1\ KO}$ (**D–F**) cells were cultured with preactivated alloreactive T cells with or without pomalidomide (10 μmol/L) as well as PD-1 mAb (10 μg/mL) for 24 hours. PD-L1 expression on tumor cells (**A** and **D**) and the percentages of dead cells (**B** and **E**) were examined by flow cytometry. The concentration of IFNγ in supernatant (**C** and **F**) was detected by ELISA. For *in vivo* tumor model, MC38^{WT} or MC38$^{PD\text{-}L1\ KO}$ tumor-bearing mice were intraperitoneally injected with pomalidomide (50 mg/kg) or vehicle of pomalidomide at day 1, 3, 6, and 8 after tumor inoculation. Tumor volume (**G**) and tumor-related survival (**H**) of MC38^{WT} or MC38$^{PD\text{-}L1\ KO}$ tumor-bearing mice in each group are shown. Data indicate mean ± SD. All samples are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *N.S.*, not significant).

growth, but eventually tumors grew progressively to kill the host. The addition of pomalidomide together with 4-1BB mAb could further retard tumor growth (Fig. 6B), as well as improve survival. As a result, 3 of 5 mice with combinatory therapy were completely tumor-free (Fig. 6C).

We also examined whether pomalidomide could enhance the antitumor response elicited by vaccine therapy (Fig. 6D). In a subcutaneous B16-OVA mouse tumor model, vaccination with OVA protein and Poly I:C had a limited effect on tumor regression when tumors became palpable (Fig. 6E). Although pomalidomide treatment alone had a limited antitumor effect, it could significantly retard tumor progression when combined with OVA vaccine (Fig. 6E). As a result, the combination of vaccine and pomalidomide extended median survival to 34 days, compared with 22 days for vaccine alone (Fig. 6F). Taken together, our results suggest that pomalidomide can be used for combinatory therapy in cancer with other immunotherapies.

Discussion

Pomalidomide and other IMiDs have long been known to have immunomodulatory functions; however, the exact molecular mechanisms behind these immunomodulatory functions are still unclear. In this study, we demonstrate through several pieces of evidence that IMiDs modulate the immune response through suppressing PD-L1 induction: (i) pomalidomide reduced PD-L1 induction on tumor cells by IFNγ or CTL; (ii) pomalidomide promoted CTL activities against tumor cells, and knockout PD-L1 on tumor cells completely eliminated this effect; (iii) in mouse tumor models, pomalidomide was able to inhibit PD-L1 upregulation on both tumor cells and host APCs, and knockout of PD-L1 on tumor cells eliminated the enhanced antitumor immunity observed with pomalidomide treatment; (iv) in a peptide-induced T-cell anergy model, pomalidomide is able to prevent peptide-induced T-cell tolerance by inhibiting PD-L1 expression on APCs. As IFNγ-induced PD-L1 upregulation on human cancer cells can be inhibited by pomalidomide, it suggests that this phenomenon is consistent between human and mouse. We believe that identification of the molecular target responsible for the immunomodulatory function of pomalidomide can help us better design new clinical application for this drug.

The molecular antitumor mechanism for IMiDs in multiple myeloma therapy has recently progressed greatly. IMiDs bind to CRBN, a substrate adapter within an E3 ubiquitin ligase complex centered on CUL4A and DDB1 (37), and results in the degradation or stabilization of target proteins (38). In human multiple myeloma, CRBN is responsible for IMiD-induced toxicity by promoting the degradation of transcriptional factors such as Ikaros (IKZF1) and Aiolos (IKZF3) by ubiquitination (21). However, there is substantial evidence suggesting IMiDs exert CRBN-independent functions. IMiDs do not interact with mouse CRBN, but there are many reports showing that IMiDs exert antitumor response in several mouse models (22, 23). In our experiments, the expression of PD-L1 was significantly inhibited by pomalidomide in cancer cells from both humans and mice. In addition, CRBN^{KO} human cancer cells lines are still responsive to IFNγ, and

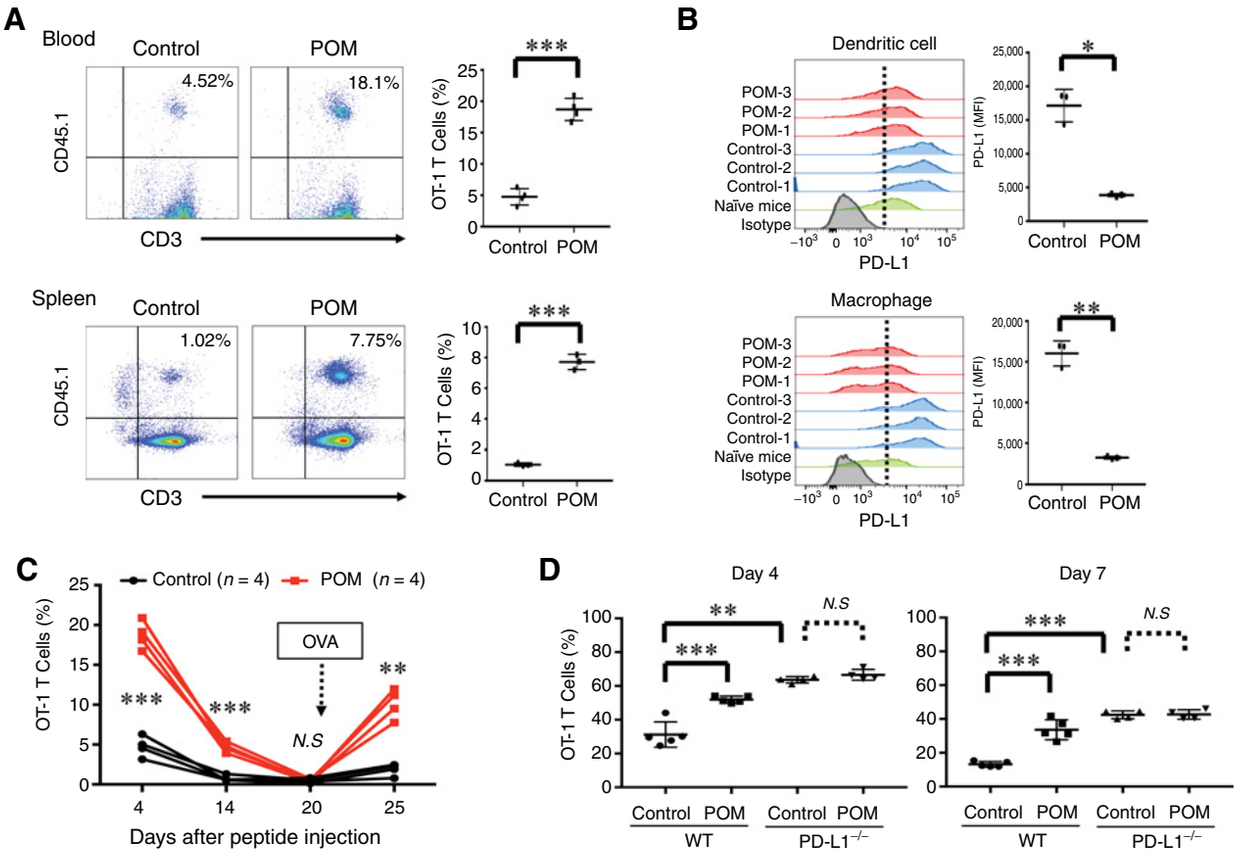

Figure 5.

Pomalidomide (POM) treatment prevents peptide-induced T-cell anergy through PD-L1. Wild-type B6 mice pretransferred with naïve OT-1 T cells were challenged with OVA peptide intravenously and followed with or without pomalidomide treatment. Mice were rechallenged with OVA peptide at day 21 after first OVA injection. **A,** The percentages of transferred $CD8^+$ OT-1 T cells in the blood or spleen samples at 4 days after peptide injection are shown. **B,** PD-L1 expression in DCs ($CD11c^+$ I-A^{b+}) and macrophages ($CD11c^-$ $CD11b^+$ I-A^{b+}) in the spleen is shown. **C,** The percentages of transferred $CD8^+$ OT-1 T-cells in peripheral blood after the first OVA peptide injection were followed by flow cytometry. The OVA peptide was reinjected 21 days after first injection. **D,** The percentages of transferred OT-1 T-cells in peripheral blood from wild-type B6 mice and PD-$L1^{-/-}$ mice with or without pomalidomide treatment at day 4 or 7 after OVA peptide injection. Data are indicated as mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *N.S.*, not significant).

pomalidomide treatment was still able to inhibit IFNγ-induced PD-L1 expression without CRBN. All these suggest that the effect of PD-L1 inhibition on cancer cells by pomalidomide does not involve CRBN.

Our results indicate that pomalidomide is not able to inhibit constitutive PD-L1 expression, but rather inhibits inducible PD-L1 expression, which mainly occurs at the transcriptional level. Because PD-1/PD-L1 blockade by mAbs can cause rare but severe cardiac toxicity in patients (39), the selectiveness of pomalidomide on inducible PD-L1 inhibitions would help to reduce this lethal risk. However, we are still not clear how pomalidomide targets to suppress PD-L1 transcription. Studies have reported that different pathways are involved to regulate constitutive and inducible PD-L1 expression (11). Inducible expression of PD-L1 on melanoma by IFNγ involves the IFNγ-JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 axis, with IRF1 binding to its promoter directly (40). Nuclear factor kappa b (NFκB) was positively correlated with inhibition of inducible PD-L1, and NFκB-binding sites were also found in the PD-L1 promoter in human monocytes after treatment with lipopolysaccharide (41). On the other hand, high constitutive expression of PD-L1 is mainly caused by changes in oncogene or tumor suppressor genes, like PTEN depletion in glioblastomas (14), and EGFR mutation in non–small cell lung cancer (42). All IMiDs are known to inhibit NFκB signaling pathway in multiple myeloma cells (43); therefore, it is possible that pomalidomide targets the NFκB pathway to inhibit inducible but not constitutive PD-L1 expression. Our studies found that pomalidomide comprehensively inhibits IFNGR signaling pathways, although the exact molecular target by pomalidomide is unclear. The potential to inhibit IFNγ-inducible PD-L1 was different among thalidomide, lenalidomide, and pomalidomide in our experiments, which is consistent with other functional aspects of these IMiDs (19).

The expression of PD-L1 can be broad, and its transcript is detected in virtually all cell types. Besides cancer cells, PD-L1 expression is found on many tumor-associated macrophages and DCs. The respective roles of host and tumor-bearing PD-L1 in tumor immunity are under extensive investigation. Early studies

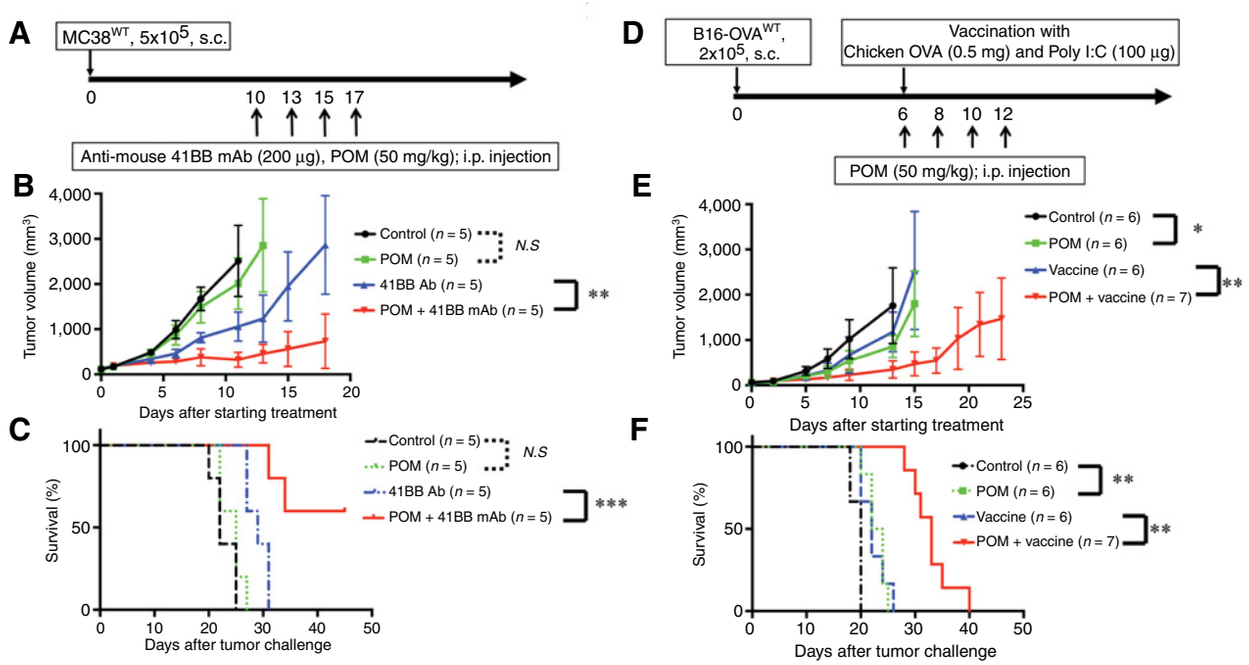

Figure 6.

Pomalidomide (POM) synergizes with other immunotherapies to treat established tumors *in vivo*. **A,** Treatment schedule of MC38 tumor-bearing mice mediated by anti-mouse 41BB mAb with pomalidomide. Tumor volume (**B**) and tumor-related survival (**C**) of MC38 tumor-bearing mice are shown. **D,** Treatment schedule of B16-OVA^{WT} tumor-bearing mice mediated by vaccine with pomalidomide. Tumor volume (**E**) and tumor-related survival (**F**) of B16-OVA^{WT} tumor-bearing mice are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *N.S.*, not significant).

have demonstrated an essential role of PD-L1 on tumor cells to evade immune attack (26, 44). The involvement of host PD-L1 signal in PD-1 therapy has been recognized recently (1, 45). The relative importance of PD-L1 on host versus tumor cells seems to depend on the level of PD-L1 expression and the immunogenicity of the respective tumor (46, 47). Our studies supported that tumor-expressing PD-L1 is required for pomalidomide-promoted antitumor immunity, while host PD-L1 is involved at a lesser extent. Tumor-associated DCs and macrophages constitutively express a high-level of PD-L1, which pomalidomide presumably has no effect on, which could be the reason why we saw a decreased role for host PD-L1 in pomalidomide-triggered antitumor immunity.

Currently IMiDs have been approved for usage in patients with multiple myeloma only; however, some clinical trials using lenalidomide or pomalidomide as a single agent or in combination with cytotoxic agents have already been reported in patients with melanoma, pancreatic cancer, hepatocellular carcinoma, and prostate cancer (48). Although antitumor response has been observed in a small population of patients with cancer in these early trials, overall efficacy is not convincing enough to lead to further trials with more patient numbers. As demonstrated in clinical therapies with immune checkpoint inhibitors, predictive biomarkers have been very helpful in selecting appropriate patients for treatment to improve overall antitumor efficacy (49). Our findings here would advocate to use PD-L1 as a prescreening biomarker to select suitable patients with cancer for pomalidomide therapy, so as to improve the antitumor efficacy. In addition, the known immunomodulatory mechanism of pomalidomide would help in designing a better combinatory strategy for pomalidomide. One interesting aspect is to reanalyze previous pomalidomide trial results based on PD-L1 expression, mutation loads, and lymphocyte infiltration. Another perspective is to start new clinical trials using PD-L1 as biomarker for pomalidomide in non-multiple myeloma cancers.

Taken together, our findings demonstrate that pomalidomide promotes antitumor immunity through suppressing PD-L1 induction and these results advocate for new therapeutic approaches for pomalidomide in cancer therapy based on PD-L1 expression. The respective contribution of immune/nonimmune antitumor effects of pomalidomide, as well as PD-L1–mediated versus non-PD-L1–mediated immunomodulation, need to be fully investigated.

Disclosure of Potential Conflicts of Interest

R.D. Schulick reports receiving a commercial research grant and has ownership interest (including stock, patents, etc.) in Aduro and Smith Kline French, and is a consultant/advisory board member for NOILE Immunte. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: Y. Fujiwara, Y. Sun, Y. Zhu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Fujiwara, Y. Sun, R.J. Torphy
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Fujiwara, Y. Sun, R.J. Torphy, J. He, Y. Zhu

Writing, review, and/or revision of the manuscript: Y. Fujiwara, Y. Sun, R.J. Torphy, K. Yanaga, R.D. Schulick, Y. Zhu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Fujiwara, Y. Sun, R.D. Schulick
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