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ANXA1-derived peptide for targeting PD-L1 degradation inhibits tumor immune evasion in multiple cancers

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ABSTRACT

Background Immune checkpoint inhibitors (ICIs) therapy targeting programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) shows promising clinical benefits. However, the relatively low response rate highlights the need to develop an alternative strategy to target PD-1/PD-L1 immune checkpoint. Our study focuses on the role and mechanism of annexin A1 (ANXA1)-derived peptide A11 degrading PD-L1 and the effect of A11 on tumor immune evasion in multiple cancers.

Methods Binding of A11 to PD-L1 was identified by biotin pull-down coupled with mass spectrometry analysis. USP7 as PD-L1's deubiquitinase was found by screening a human deubiquitinase cDNA library. The role and mechanism of A11 competing with USP7 to degrade PD-L1 were analyzed. The capability to enhance the T cell-mediated tumor cell killing activity and antitumor effect of A11 via suppressing tumor immune evasion were investigated. The synergistic antitumor effect of A11 and PD-L1 mAb (monoclonal antibody) via suppressing tumor immune evasion were also studied in mice. The expression and clinical significance of USP7 and PD-L1 in cancer tissues were evaluated by immunohistochemistry.

Results A11 decreases PD-L1 protein stability and levels by ubiquitin proteasome pathway in breast cancer, lung cancer and melanoma cells. Mechanistically, A11 competes with PD-L1's deubiquitinase USP7 for binding PD-L1, and then degrades PD-L1 by inhibiting USP7-mediated PD-L1 deubiquitination. Functionally, A11 promotes T cell ability of killing cancer cells in vitro, inhibits tumor immune evasion in mice via increasing the population and activation of $CD8^+$ T cells in tumor microenvironment, and A11 and PD-1 mAb possess synergistic antitumor effect in mice. Moreover, expression levels of both USP7 and PD-L1 are significantly higher in breast cancer, non-small cell lung cancer and skin melanoma tissues than those in their corresponding normal tissues and are positively correlated in cancer tissues, and both proteins for predicting efficacy of PD-1 mAb immunotherapy and patient prognosis are superior to individual protein.

Conclusion Our results reveal that A11 competes with USP7 to bind and degrade PD-L1 in cancer cells, A11 exhibits obvious antitumor effects and synergistic antitumor activity with PD-1 mAb via inhibiting tumor immune evasion and A11 can serve as an alternative strategy for ICIs therapy in multiple cancers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint inhibitors (ICIs) therapy targeting programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) displays significant clinical benefits for treating multiple advanced cancers. However, fewer than 30% of cases across multiple types of cancers are responsive to ICIs therapy, highlighting the need to develop a new strategy to target PD-1/PD-L1 immune checkpoint.

WHAT THIS STUDY ADDS

⇒ Annexin A1-derived peptide A11 competes with USP7 to bind and degrade PD-L1 in cancer cells and exhibits obvious antitumor effects and synergistic antitumor activity with PD-1 mAb via inhibiting tumor immune evasion in multiple cancers.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ A11 targeting PD-L1 degradation represents a potential new strategy for cancer immunotherapy.

BACKGROUND

The programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) interaction is an important immune checkpoint mediating tumor immunosuppression that has become one of the major targets in cancer immunotherapy.[1] This immune checkpoint suppresses T cell activation and function and prevents cytotoxic T cells from killing tumor cells, resulting in tumor immune escape.[2] A major feature of tumor immune evasion is the expression of multiple inhibitory ligands, notably PD-L1 on the surface of cancer cells,[3] and PD-L1 interacts with PD-1 on the surface of T cells, which acts as a brake for antitumor immunity. Moreover, patients with expression of PD-L1 on cancer cells prefer to respond to anti-PD-1/PD-L1 antibody therapy.[4] Hence, it is critical to discover the strategies regulating the expression and stability of PD-L1.

Immune checkpoint inhibitors (ICIs) therapy using anti-PD-1/PD-L1 antibodies reactivates inactivated T cells and recovers cytotoxic T cells to kill tumor cells and has displayed significant clinical benefits for treating multiple advanced cancers.[5–7] However, accumulated data showed that fewer than 30% of cases across multiple types of cancers are responsive to ICIs therapy, and a significant portion of cancer patients do not benefit from ICIs therapy.[6] Moreover, ICIs therapy using anti-PD-1/PD-L1 antibodies display several limitations, for example, immune-related adverse events (irAEs), low permeability, immunogenicity and complex production process. Therefore, developing an alternative strategy to target PD-1/PD-L1 immune checkpoint will bring new options for cancer immunotherapy.

Ubiquitination is one of the most common pathways for protein degradation. Following ubiquitin modification, proteins are usually degraded by proteasomes. Correspondingly, there is a deubiquitinating enzyme system that can regulate protein stability by removing ubiquitin chains. A growing amount of evidence suggests that ubiquitination and deubiquitination of PD-1 and PD-L1 play crucial roles in the regulation of their stabilization and levels.[8] The abnormal ubiquitination and deubiquitination of PD-1/PD-L1 influence PD-1/PD-L1-mediated tumor immunosuppression. Emerging evidence highlights the importance of deubiquitinases (DUBs) in modulating PD-L1 stability and tumor immune escape.[9–11]

Annexin A1 (ANXA1) is a Ca^{2+}-dependent phospholipid-binding protein.[12] It plays a role in the immune response as effector of glucocorticoid-mediated responses and regulator of inflammatory process.[13–15] ANXA1, first described in the context of inflammation, appears to be deregulated in various cancers, which has been linked to tumor development and metastasis.[16–18] Our previous study showed that ANXA1-derived peptide (EYVQTVKSSKG), named as A11 (ANXA1-derived 11 amino acid-long peptide), dramatically suppresses nasopharyngeal carcinoma cell growth in vitro and in immune-deficient nude mice by degrading EphA2 protein.[19] To further reveal the antitumor mechanism of A11 peptide, we recently used biotin pull-down and mass spectrometry analysis to search proteins interacting with A11 peptide in cancer cells and found PD-L1 interacting with A11. However, the function and significance of A11 bound to PD-L1 are completely unclear.

In this work, we investigated the effects of A11 on PD-L1 protein stability and tumor immunosuppression, and found that A11 competed with deubiquitinase USP7 for binding PD-L1 and degraded PD-L1 in multiple cancer cells. A11 sensitized cancer cells to T cell-mediated killing *in vitro*, and dramatically inhibited the growth of cancer cells in immune-competent mice via suppressing tumor immune escape, and A11 and PD-1 mAb displayed synergistic antitumor activity in mice. These findings suggest that A11 can serves as an alternative strategy for cancer ICIs therapy.

MATERIALS AND METHODS

Human tissue specimens

One hundred and thirty-two cancer tissues from the patients with breast cancer, NSCLC (non-small cell lung cancer) or skin cutaneous melanoma, who received PD-1 mAb (Nivolumab) treatment between January 2016 and January 2018, were collected from Xiangya Hospital of Central South University. Detailed clinicopathologic information is presented in online supplemental tables S1–S3. Tumor response to PD-1 mAb treatment was assessed as per the Response Evaluation Criteria in Solid Tumors, V.1.1 (RECIST V.1.1).[20] For details, see the online supplemental materials and methods section.

Animal experiments

Female C57BL/6 and BALB/c immune-competent mice and female nude mice (BALB/c nu/nu) that were 5 weeks old were obtained from the Experimental Animal Center of Central South University and maintained in pathogen-free conditions. The antitumor effects of A11 or/and PD-1mAb were tested in mice. For details, see the online supplemental materials and methods.

Biotin pull-down coupled with mass spectrometry analysis

Biotin pull-down coupled with mass spectrometry analysis was preformed to search proteins that interact with A11 peptide in cancer cells. For details, see the online supplemental materials and methods.

Tumor-infiltrating lymphocyte profile analysis by flow cytometry

Tumor-infiltrating lymphocyte profile of implanted tumors were analyzed by flow cytometry as described previously.[3 21] For details, see the online supplemental materials and methods.

T cell-mediated tumor cell killing assay

T cell-mediated tumor cell killing assay was performed to detect the effect of A11 on tumor immune escape as previously described.[9 22 23] For details, see the online supplemental materials and methods.

Apoptotic and activity assay of Jurkat T cells cocultured with cancer cells

The effect of A11 on apoptosis and activity of Jurkat T cells cocultured with cancer cells as previously described.[24] For details, see the online supplemental materials and methods.

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed to detect protein–protein interaction and PD-L1 ubiquitination. In brief, whole cell lysates were precleared with Protein A/G–Sepharose 4B for 2 hours, and then incubated with indicated antibodies or isotype control IgG and Protein A/G-Sepharose 4B overnight at 4°C. After five times wash with RIPA buffer, beads were boiled in 2×SDS-PAGE loading buffer for 5 min to elute protein complexes,

followed by SDS-PAGE separation and immunoblotting with specific antibodies.

Duolink proximity ligation assay

Proximity ligation assay was performed to detect the interaction between USP7 and PD-L1 using Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101; Sigma-Aldrich) as previously described.[25] For details, see the online supplemental materials and methods.

Western blot

Immunoblotting was performed as described previously by us.[19] For details, see the online supplemental materials and methods.

Quantitative real-time PCR

Real-time qRT-PCR was performed to detect the expression of *CD274* in the indicated cells. The primers are presented in online supplemental table S4. For details, see the online supplemental materials and methods.

Immunofluorescent staining

PD-L1, CD8a and granzyme B (GZMB) in the implanted tumors from immune-competent mice was performed as described previously.[9] For details, see the online supplemental materials and methods.

Immunohistochemical staining and staining evaluation

Immunohistochemistry and staining evaluation of USP7 and PD-L1 were performed on the formalin-fixed and paraffin-embedded tissue sections. For details, see the online supplemental materials and methods.

Molecular docking

Molecular docking of PD-L1-USP7 and PD-L1-A11 was performed as described previously.[26–28] For details, see the online supplemental materials and methods.

Statistical analysis

All analysis was performed using IBM SPSS statistical software package V.22, and data visualization was generated by GraphPad Prism V.7.0. Data are expressed as the mean±SD. For comparisons between two groups, a Student's t-test was used, and for analysis with multiple comparisons, one-way analysis of variance (ANOVA), followed by Turkey's post hoc analysis was used. Tumor growth curves were assessed by two-way ANOVA with Turkey's post hoc analysis for multiple comparisons. Correlations were analyzed using the Pearson correlation test. Classification variables were compared by χ^2 test. Survival curves were obtained by using the Kaplan-Meier method, and comparisons were made by using log-rank test. Cox proportional hazards regression analysis was used to analyze the effect of clinical variables on patient survival. P values <0.05 were considered statistically significant.

RESULTS

Binding of A11 peptide with PD-L1 decreases PD-L1 stability by ubiquitin proteasome pathway in multiple cancer cells

To search proteins that interact with A11, proteins were pulled down with biotin-labeled A11 from breast cancer cell extracts, separated on SDS-PAGE and stained with Coomassie blue (online supplemental figure S1A). All protein bands pulled down by A11 were subjected to LC-MS/MS analysis, and a total of 50 proteins including PD-L1 were identified (online supplemental figure S1B and online supplemental table S5). As previous studies showed that peptides interacting with PD-L1 could inhibit cancer immune evasion via blocking interaction of PD-L1 and PD-1,[29–31] binding of A11 with PD-L1 was further investigated. Biotin pull-down assay confirmed that A11 bound to PD-L1 in the human and mouse breast cancer, lung cancer and melanoma cells (online supplemental figure S1C,D) and also bound to exogenous PD-L1 in HEK293 cells (online supplemental figure S1E). Collectively, these data provide strong evidences for binding of A11 with PD-L1 in multiple cancer cells.

To explore the role of A11 binding to PD-L1 in cancer cells, A11 peptide was synthesized in fusion to previously characterized cell-penetrating peptide (CPP) (YGRKKRRQRRR), [32] thereafter named as CPP-A11, and CPP was used as control. Efficient cellular uptake of both peptides was confirmed by immunofluorescent labeling with FITC (figure 1A). Western blot showed that CPP-A11 dramatically decreased PD-L1 expression in multiple human and mouse cancer cells in a dose-dependent manner (figure 1B,C) and also decreased exogenous PD-L1 expression in HEK239 cells (online supplemental figure S2A). Flow cytometric analysis showed that CPP-A11 obviously decreased PD-L1 expression on the surface of cancer cells (figure 1D). CPP-A11 also downregulated IFN-gama-induced PD-L1 expression in multiple cancer cells (online supplemental figure S2B). Moreover, both Western blot and immunofluorescent staining showed that CPP-A11 dramatically decreased PD-L1 levels in the membrane and cytoplasm of cancer cells (online supplemental figure S3A,B). Collectively, these data demonstrate that A11 decreases PD-L1 expression in multiple human and mouse cancer cells.

Next, we analyzed the effect of CPP-A11 on PD-L1 protein stability after blocking protein synthesis with cycloheximide and observed that PD-L1 was degraded in the CPP-A11-treated human breast cancer, lung cancer and melanoma cells (figure 1E), but had no change in its mRNA level (online supplemental figure S4), indicating that A11 tightly controlled PD-L1 protein stability. Moreover, decrease of PD-L1 protein in the CPP-A11-treated cancer cells was specifically reversed by proteasome inhibitor MG132 (figure 1F), indicating a proteasome-dependent mechanism in PD-L1 destabilization by A11. We then investigated how A11 destabilizes PD-L1. As ubiquitination is a key mechanism driving the proteasomal degradation of PD-L1, we assessed the capacity of A11 to modulate PD-L1 ubiquitination and observed that CPP-A11 increased PD-L1 polyubiquitination in the three types of human cancer cells (figure 1G), indicating that A11 decreases PD-L1 stabilization by promoting its polyubiquitination and degradation. Moreover, we examined the composition of A11-promoted PD-L1 polyubiquitin

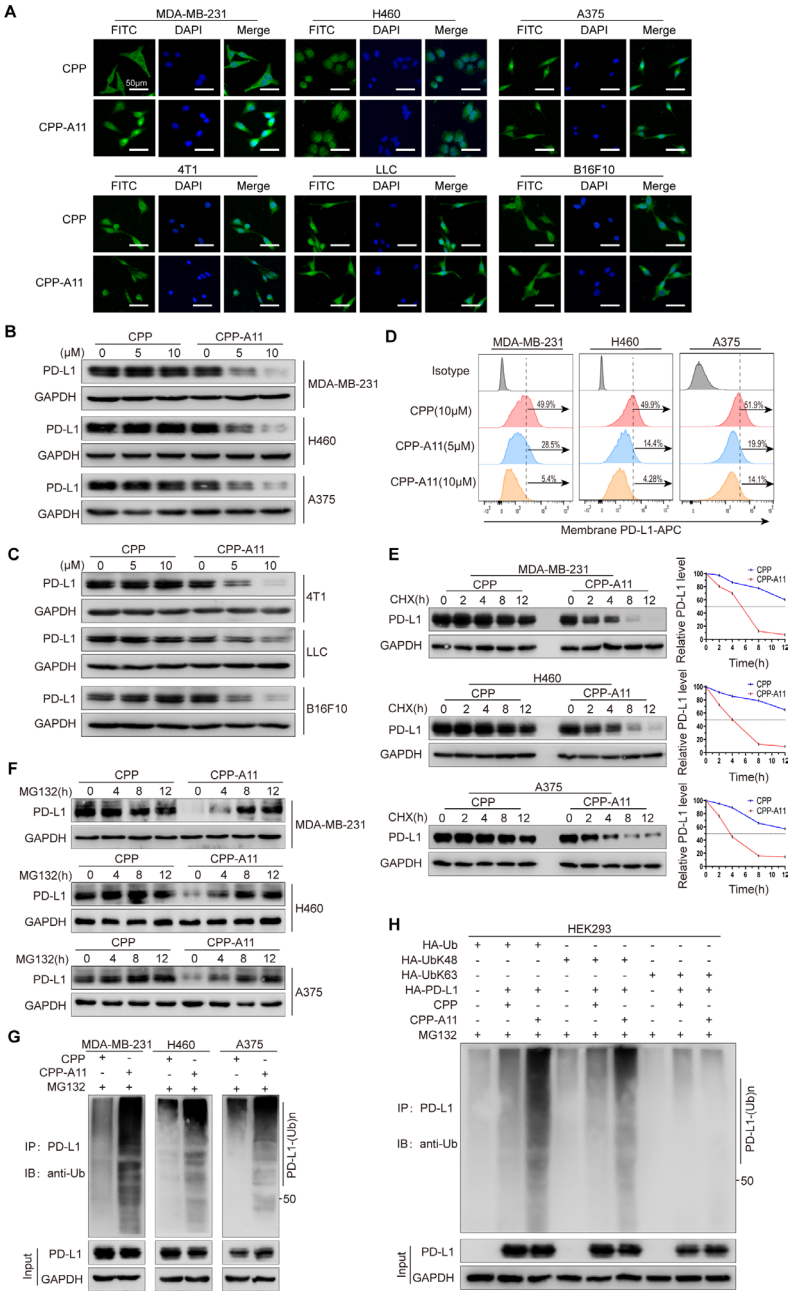

Figure 1 Binding of A11 peptide to PD-L1 decreases PD-L1 stability by ubiquitin proteasome pathway in multiple cancer cells. (A) Cellular uptake of CPP-A11 or CPP peptide. The human breast cancer MDA-MB-231, lung cancer H460 and melanoma A375 cells (*top*), and mouse breast cancer 4T1, lung cancer LLC and melanoma B16F10 cells (*bottom*) were incubated with FITC labeled 10 μM CPP-A11 or CPP for 24 hours and observed by fluorescent microscopy. Cell nuclei were stained by DAPI. Scale bars=50 μm. (B–D) A11 decreases PD-L1 protein levels. Cancer cells were treated with indicated concentration of CPP-A11 or CPP for 24 hours and subjected to further analysis. Western blot showing that A11 decreases PD-L1 levels in the human MDA-MB-231, H460 and A375 cells (B), and mouse 4T1, LLC and B16F10 cells (C) and flow cytometric analysis showing that A11 decreases PD-L1 levels on the surface of human MDA-MB-231, H460 and A375 cells (D). (E–H) A11 decreases PD-L1 stability by ubiquitin proteasome pathway. (E) Western blot showing the effect of A11 on PD-L1 protein stability in the human MDA-MB-231, H460 and A375 cells treated with 20 μg/mL cycloheximide (CHX) for indicated times. (F) Western blot showing reversion of PD-L1 protein levels by proteasome inhibitor MG132 in the A11-treated human MDA-MB-231, H460 and A375 cells. Cancer cells were treated with 10 μM CPP-A11 or CPP for 24 hours, followed by 10 μM MG132 treatment for indicated times. (G) A11 increases PD-L1 polyubiquitination in the human MDA-MB-231, H460 and A375 cells. Cancer cells were treated with 10 μM CPP-A11 or CPP for 24 hours and 10 μM MG132 for another 12 hours, and subjected to immunoprecipitation analysis with anti-PD-L1 antibody followed by immunoblotting with antipolyubiquitin antibody. (H) Type of A11-increased PD-L1 polyubiquitination. HEK293 cells were transfected with indicated plasmids for 48 hours, treated with 10 μM CPP-A11 or CPP for 24 hours and 10 μM MG132 for another 12 hours, and subjected to immunoprecipitation analysis with anti-PD-L1 antibody followed by immunoblotting with antipolyubiquitin antibody. IP, immunoprecipitation; IB, immunoblotting; PD-L1, programmed cell death ligand 1.

chains in HEK293 cells and observed that the total and K48-linked ubiquitination but not the K63-linked ubiquitination of PD-L1 was obviously upregulated by CPP-A11 (figure 1H), suggesting that A11 promotes the K48-linked polyubiquitination of PD-L1. Together, these data demonstrate that binding of A11 with PD-L1 decreases PD-L1 stability by ubiquitin proteasome pathway in multiple cancer cells.

USP7 is the deubiquitinase of PD-L1 in multiple cancer cells

Accumulative studies indicate importance of deubiquitinases (DUBs) in modulating PD-L1 stability.[9–11] Therefore, we screened PD-L1's DUB by using a human DUB cDNA library and identified USP7 as an inducer of PD-L1 expression in HEK293 cells (online supplemental figure S5A). Both Co-IP and Duolink in situ proximity ligation assay showed that USP7 physically interacted with PD-L1 (online supplemental figure S5B, C) in the human breast cancer, lung cancer and melanoma cells. Knockdown of USP7 by siRNAs significantly decreased PD-L1 protein levels (online supplemental figure S5D) and increased PD-L1 ubiquitination levels in the three types of human cancer cells (online supplemental figure S5E). Moreover, USP7 significantly upregulated exogenous PD-L1 levels and decreased its polyubiquitination levels in the HEK293 cells cotransfected with USP7 and PD-L1 expression plasmids (online supplemental figure S5F,G). However, catalytically inactive mutant USP7 CS (C223S)[33] did not influence the expression and ubiquitination levels of exogenous PD-L1 in the HEK293 cells (online supplemental figure S5F,G). Collectively, these data provide strong evidences for USP7 as the deubiquitinase of PD-L1 in multiple cancer cells.

A11 peptide competes with USP7 for binding PD-L1 to decrease its stability in multiple cancer cells

Our results showed that A11 bound and downregulated PD-L1, whereas USP7 bound and upregulated PD-L1 in multiple cancer cells. Therefore, we reasonably speculated that A11 competes with USP7 for binding PD-L1 to decrease its levels. Molecular docking showed that biding of both USP7 TRAF domain (Y143 and K148) and A11 with PD-L1 intercellular domain (ICD) (D268 and T277), *i.e.* that PD-L1 binding interface of USP7 and A11 is mutually exclusive (online supplemental figure S6), indicating that A11 competes with USP7 for binding PD-L1. To confirm the results of molecular docking, we constructed deletion mutants of PD-L1 and USP7 (figure 2A) and cotransfected wild-type (WT) or ICD deletion PD-L1 with WT USP7 into HEK293 cells following Co-IP analysis. The results showed that ICD deletion PD-L1 (D260-290) could not bind to USP7 (figure 2B), indicating PD-L1 ICD responsible for binding USP7. Biotin pull-down assay showed that A11 could not bind with ICD deletion PD-L1 (figure 2C), indicating PD-L1 ICD responsible for binding A11. We also cotransfected WT or deletion mutants of USP7 with WT PD-L1 into HEK293 cells following Co-IP analysis. The results showed that TRAF deletion USP7 (D22-208) could not bind to PD-L1 (figure 2D), indicating USP7 TRAF responsible for binding PD-L1. These results are in agreement with the results of molecular docking, supporting a model in which both USP7 and A11 bind to the ICD of PD-L1. Moreover, Co-IP showed that CPP-A11 dramatically decreased USP7 bound to PD-L1 in the human breast cancer, lung cancer and melanoma cells (figure 2E), as well as decreased exogenous USP7 bound to exogenous PD-L1 in HEK293 cells (figure 2F). Together, these data demonstrate that A11 competes with USP7 for binding PD-L1.

Next, we analyzed whether competition of A11 with USP7 for binding PD-L1 inhibits USP7-mediated PD-L1 deubiquitination and reduces its levels. Co-IP showed that CPP-A11 obviously antagonized USP7-downregulated endogenous PD-L1 ubiquitination in the three types of human cancer cells (figure 2G) and also antagonized exogenous USP7-downregulated exogenous PD-L1 ubiquitination in HEK293 cells (figure 2H). Western blot showed that CPP-A11 obviously inhibited USP7-upregulated endogenous PD-L1 levels in the three types of human cancer cells (figure 2I) and also inhibited exogenous USP7-upregulated exogenous PD-L1 levels in HEK293 cells (figure 2J). Together, these data demonstrate that A11 degrades PD-L1 by competing with USP7 for binding PD-L1 in multiple cancer cells.

An intact immune system increases the antitumor effect of A11 peptide in mice

As A11 degrades PD-L1 in multiple cancer cells, we assessed whether an intact immune system increases A11 antitumor activity in mice. Mouse 4T1 breast cancer, LLC lung cancer or B16F10 melanoma cells were subcutaneously inoculated into immune-deficient and immune-competent mice, respectively, and tumor-bearing mice received treatment of CPP-A11 or control CPP peptide via peritoneal injection as indicated in online supplemental figure S7A. The result showed that antitumor effect of CPP-A11 in immune-competent mice was much stronger than that in immune-deficient mice (online supplemental figure S7B–D). Moreover, immunohistochemistry (IHC) showed that PD-L1 expression markedly decreased in the implanted tumors of mice received CPP-A11 treatment (online supplemental figure S7E). The results reveal that an intact immune system increases the antitumor effect of A11, which may be attributed to PD-L1 degradation.

A11 peptide promotes T cell ability of killing cancer cells in vitro

Binding of PD-L1 on tumor cells with PD-1 on T cells inhibits effector function of T cells.[3 21] Therefore, we assessed the effect of A11 on T cell ability of killing cancer cells using T cell-mediated tumor cell killing assay as previously described.[9 22 23] Human cancer cells were cultured alone or cocultured with activated human T cells in presence of CPP-A11 or control CPP. The results showed that CPP-A11 sensitized cancer cells to T cell-mediated killing as compared with CPP (figure 3A), and PD-L1

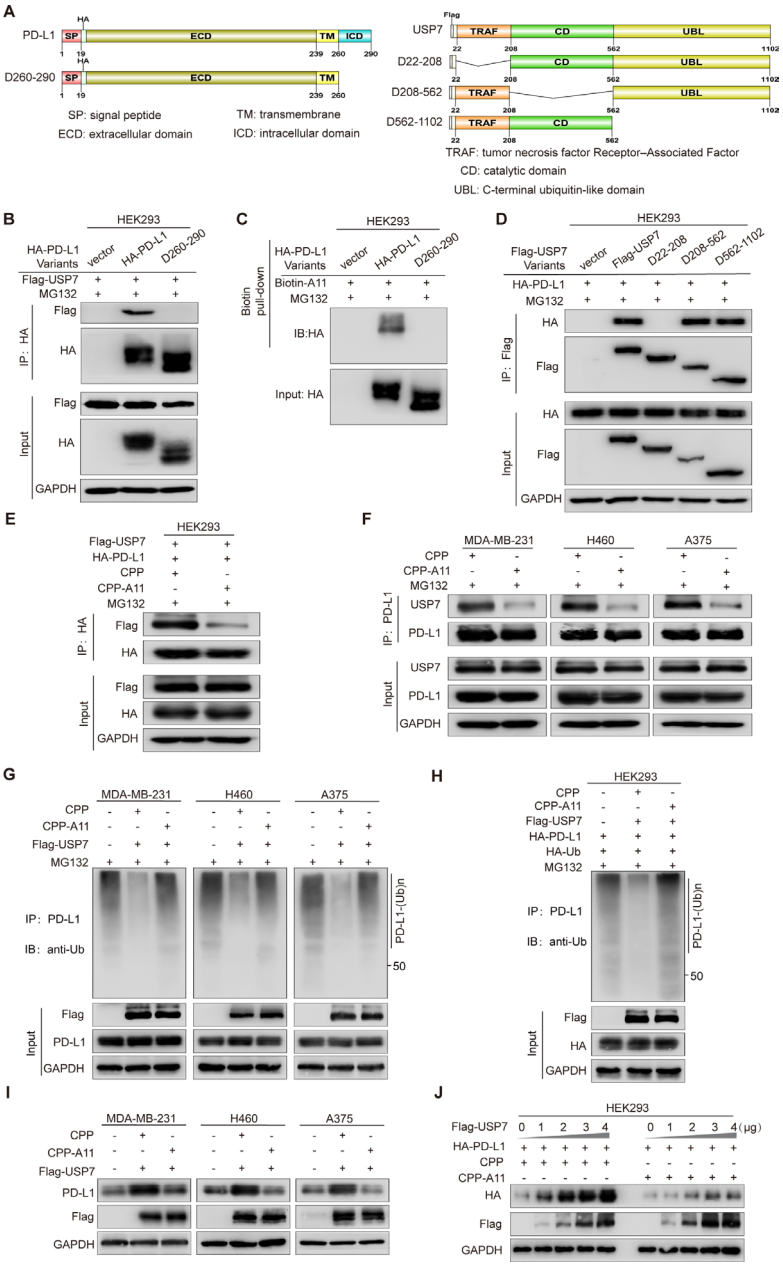

Figure 2 A11 peptide competes with USP7 for binding PD-L1 to decrease its stability in multiple cancer cells. (A–D) Mapping of binding region of A11 and USP7 with PD-L1. (A) Diagrammatic representation of PD-L1, USP7 and their deleted forms. The main regions of both proteins are indicated. Numbers indicate amino acid position within the sequence. (B) Co-IP showing the region of PD-L1 bound to USP7. Total cell proteins from HEK293 cells transfected with indicated constructs were subjected to immunoprecipitation with anti-HA (PD-L1), followed by immunoblotting with anti-FLAG (USP7) antibody. (C) Biotin pull-down showing the region of PD-L1 bound to A11. Total cell proteins from HEK293 cells transfected with indicated constructs were subjected to biotin pull-down using biotin-labeled A11, followed by immunoblotting with anti-HA (PD-L1) antibody. (D) Co-IP showing the region of USP7 bound to PD-L1. Total cell proteins from HEK293 cells transfected with indicated constructs were subjected to immunoprecipitation with anti-FLAG (USP7) antibody, followed by immunoblotting with anti-HA (PD-L1) antibody. (E and F) Competition of A11 and USP7 for binding PD-L1. (E) Co-IP showing that A11 decreases USP7 bound to PD-L1 in the human MDA-MB-231, H460 and A375 cancer cells. (F) Co-IP showing that A11 decreases exogenous USP7 bound to exogenous PD-L1 in the HEK293 cells co-transfected with USP7 and PD-L1 expression plasmids. (G–J) A11 degrades PD-L1 by competing with USP7 for binding PD-L1. Cells were transfected with indicated plasmid for 48 hours, followed by treatment with 10 μM CPP-A11 or CPP for another 24 hours and subjected to Co-IP or Western blot analysis. (G) Co-IP showing that A11 antagonizes USP7-downregulated endogenous PD-L1 ubiquitination in the human MDA-MB-231, H460 and A375 cancer cells transfected with USP7 expression plasmid. (H) Co-IP showing that A11 antagonizes USP7-downregulated exogenous PD-L1 ubiquitination in the HEK293 cotransfected with USP7 and PD-L1 expression plasmids. (I) Western blot showing A11 antagonizes USP7-upregulated endogenous PD-L1 levels in the human MDA-MB-231, H460 and A375 cancer cells transfected with USP7 expression plasmid. (J) Western blot showing A11 antagonizes USP7-upregulated exogenous PD-L1 expression in the HEK293 cells transfected with USP7 and PD-L1 expression plasmids. D, deletion; PD-L1, programmed cell death ligand 1.

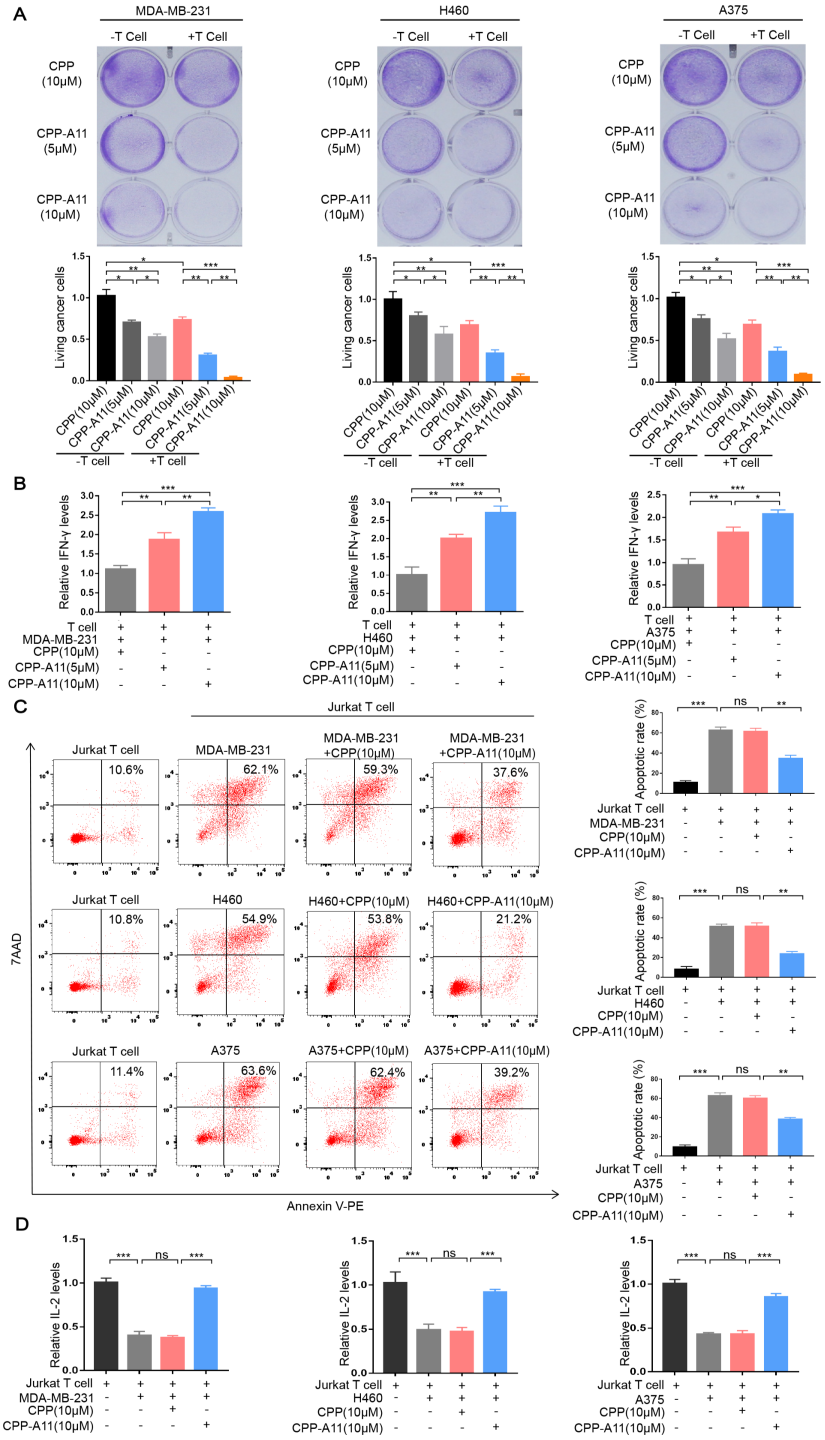

Figure 3 A11 peptide promotes T cell ability of killing cancer cells in vitro. (A) The effect of A11 on T cell killing of cancer cells. Human MDA-MB-231, H460 or A375 cancer cells were cultured alone or cocultured with activated human T cells in the presence of CPP-A11 or CPP for 48 hours, and the survived cancer cells were subjected to crystal violet staining. The ratio of cancer cells to T cells is 1:3. Representative images are shown on the top, and quantitative data are presented on the bottom. (B) Histograms showing the levels of IFN-γ secreted by activated T cells cocultured with MDA-MB-231, H460 or A375 cancer cells in the presence of CPP-A11 or CPP for 48 hours. (C) The effect of A11 on apoptosis of Jurkat T cells cocultured with cancer cells. Activated Jurkat T cells were cultured alone or cocultured with human MDA-MB-231, H460 or A375 cancer cells in the presence of CPP-A11 or CPP for 24 hours, and apoptosis of cells was detected by flow cytometry. The ratio of cancer cells to Jurkat T cells is 1:5. Representative images are shown on the left, and quantitative data are presented on the right. Apoptosis of Jurkat T cells cultured alone and without CPP-A11 treatment served as control. (D) Histograms showing the levels of IL-2 secreted by Jurkat T cells cocultured with human MDA-MB-231, H460 and A375 cancer cells in the presence of CPP-A11 or CPP for 24 hours. The levels of IL-2 secreted by Jurkat T cells cultured alone and without CPP-A11 treatment served as control. n=3. Data represent mean±SD. Statistical differences were determined by one-way analysis of variance. *P<0.05; **p<0.01; ***p<0.001; NS, no significance.

overexpression in cancer cells could rescue the survival of CPP-A11-treated cancer cells cocultured with activated human T cells (online supplemental figure S8), indicating that A11 increases T cell ability of killing cancer cells via targeting PD-L1 degradation. IFN-γ and IL-2 are cytokines produced by activated T cells and promotes cytotoxic activity of $CD8^+$ T cells.[34 35] We also observed that coculture with cancer cells decreased levels of IFN-γ secreted by activated T cells, which could be reversed by CPP-A11 (figure 3B). Furthermore, we assessed the effect of A11 on apoptosis and activity of Jurkat T cells cocultured with cancer cells as previously described.[24] Flow cytometric analysis showed that coculture with cancer cells increased apoptotic rate of Jurkat T cells, which could be recovered by CPP-A11 (figure 3C). We also observed that coculture with cancer cells reduced levels of IL-2 secreted by activated Jurkat T cells, which could be reversed by CPP-A11 (figure 3D). Collectively, these data indicate that A11 promotes T cell ability of killing cancer cells in vitro.

Antitumor effect of A11 alone and combining with PD-1 mAb in immune-competent mice

Next, we further assessed the antitumor effect of A11 alone and combining with PD-1 mAb in immune-competent mice. Mouse 4T1, LLC and B16F10 cells were subcutaneously inoculated into immune-competent mice, respectively. Seven days after inoculation, tumor-bearing mice received treatment of CPP-A11 and/or PD-1 mAb (BE0146; BioXcell) via peritoneal injection as indicated in figure 4A, and control mice received the peritoneal injection of both CPP and rat IgG2a (BE0089; BioXcell). Twelve days after initial treatment, we assessed tumor suppression function of CPP-A11 and/or PD-1 mAb and observed that CPP-A11 inhibited the implanted tumor growth of 4T1, LLC and B16F10 cancer cells and extended the survival of tumor-bearing mice (figure 4B–D), whereas PD-1 mAb only inhibited the implanted tumor growth of LLC cells and extended the survival of mice with the implanted tumors of LLC cells (figure 4B–D). Implanted tumors of 4T1 and B16F10 cells were resistant to PD-1 mAb treatment (figure 4B–D), which is consistent with the previous reports.[36–38] Moreover, cotreatment with CPP-A11 and PD-1 mAb conferred better efficacy and longer survival as compared with CPP-A11 and PD-1 mAb treatment alone in the three types of tumor-bearing mice (figure 4B–D). Collectively, these results demonstrate that A11 possesses obvious antitumor effect and synergistic antitumor activity with PD-1 mAb in immune-competent mice.

A11 alone and combining with PD-1 mAb inhibit tumor immune escape in immune-competent mice

To assess the effect of A11 alone and combining with PD-1 mAb on tumor immune escape, fluorescence-activated cell sorting (FACS) and immunofluorescent staining were performed to detect the number and activity of infiltrating $CD8^+$ T cells in the implanted tumors of 4T1, LLC and B16F10 cells received treatment of CPP-A11 and/or PD-1 mAb. FACS showed that CPP-A11 treatment significantly increased the population of $CD8^+$ T cells and $CD8^+$granzyme B $(GZMB)^+$ T cells in the three types of implanted tumors (figure 5A–F), whereas PD-1 mAb treatment only increased the population of $CD8^+$ T cells and $CD8^+GZMB^+$ T cells in the implanted tumors of LLC cells (figure 5A–F). Cotreatment with CPP-A11 and PD-1 mAb displayed a synergetic effect in increasing the population of $CD8^+$ T cells and $CD8^+GZMB^+$ T cells in the three types of implanted tumors (figure 5A–F). Immunofluorescent staining showed that CPP-A11 treatment obviously decreased PD-L1 levels and significantly increased the density of $CD8^+$ T cells and $CD8^+GZMB^+$ T cells in the three types of implanted tumors (figure 6A–F), whereas PD-1 mAb treatment only increased the density of $CD8^+$ T cells and $CD8^+GZMB^+$ T cells in the implanted tumors of LLC cells (figure 6A–F). Cotreatment with CPP-A11 and PD-1 mAb displayed a synergetic effect in increasing the density of $CD8^+$ T cells and $CD8^+GZMB^+$ T cells in the three types of implanted tumors (figure 6A–F). Collectively, these results demonstrate that A11 inhibits tumor immune escape in immune-competent mice, and cotreatment with A11 and PD-1 mAb displays synergistic effect in suppressing tumor immune escape.

USP7 and PD-L1 expression levels are correlated with efficacy of PD-1 mAb treatment and prognosis in patients with breast cancer, lung cancer or melanoma

To investigate the clinical significance of USP7 as the deubiquitinase of PD-L1, we used IHC to detect expression levels of USP7 and PD-L1 in 132 tumor tissues from patients with breast cancer, NSCLC or skin cutaneous melanoma, who received PD-L1 mAb treatment, and their corresponding 20 normal tissues were used as control. The clinicopathological characteristics of the patients are shown in online supplemental tables S1 and S2. IHC showed that expression levels of both USP7 and PD-L1 in the cancer tissues were significantly higher than those in the normal tissues (online supplemental figure S9), and USP7 levels were positively correlated with PD-L1 levels in cancer tissues(figure 7A,B), supporting USP7 as the deubiquitinase of PD-L1. We further assessed correlation of USP7 and PD-L1 expression levels with efficacy of PD-1 mAb therapy in these patients. In the 132 patients received PD-1 mAb treatment, 41 and 91 patients were classified as responders and non-responders (online supplemental tables S1 and S2). There were not significant differences in the age, gender, tumor type, TNM stage and prior systemic therapies between the two groups (online supplemental table S3). All the patients' response to PD-1 mAb therapy was evaluated by RECIST V.1.1. The results showed that 7 of 46 patients with low USP7 expression and 34 of 86 patients with high USP7 expression achieved complete remission (CR) or partial remission (PR) (figure 7C), and 4 of 51 patients with low PD-L1 expression and 37 of 81 patients with high PD-L1-expression achieved CR or PR (figure 7D). Objective response rate (ORR) in patients with high levels of USP7/

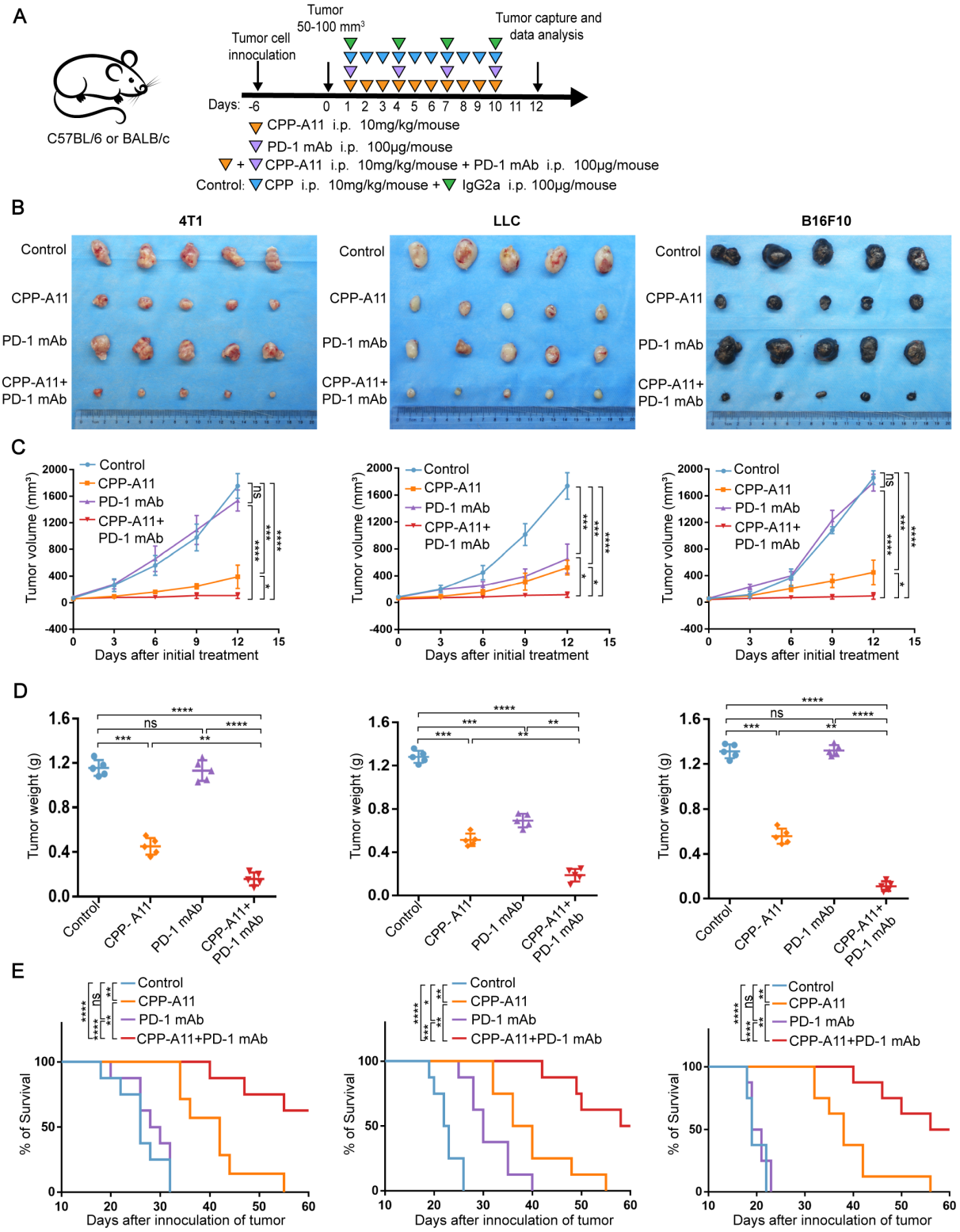

Figure 4 Antitumor effect of A11 alone and combining with PD-1 mAb in immune-competent mice. (A) A schematic diagram illustrating the treatment plan of A11 or/and PD-1 mAb in the immune-competent mice with the implanted tumors of 4T1, LLC or B16F10 cells. (B) The photographs of xenograft tumors of 4T1, LLC and B16F10 cancer cells 12 days after initial treatment. (C) The growth curves of xenograft tumors of 4T1 (*left*), LLC (*middle*) and B16F10 (*right*) cells after initial treatment. Statistical differences were determined by two-way analysis of variance (ANOVA), followed by Turkey's post hoc analysis. (D) Summary of weight data of xenograft tumors of 4T1 (*left*), LLC (*middle*) and B16F10 (*right*) cells 12 days after initial treatment. Statistical differences were determined by one-way ANOVA. (E) Kaplan-Meier survival curves for mice with xenograft tumors of 4T1 (*left*), LLC (*middle*) and B16F10 (*right*) cells and treated with A11 peptide or/and PD-1 mAb (n=8 mice per group). data represent means±SD. Statistical differences were determined by log-rank test. *P<0.05; **p<0.01; ***p<0.001; ****, p<0.0001; NS, no significance; PD-1, programmed cell death 1.

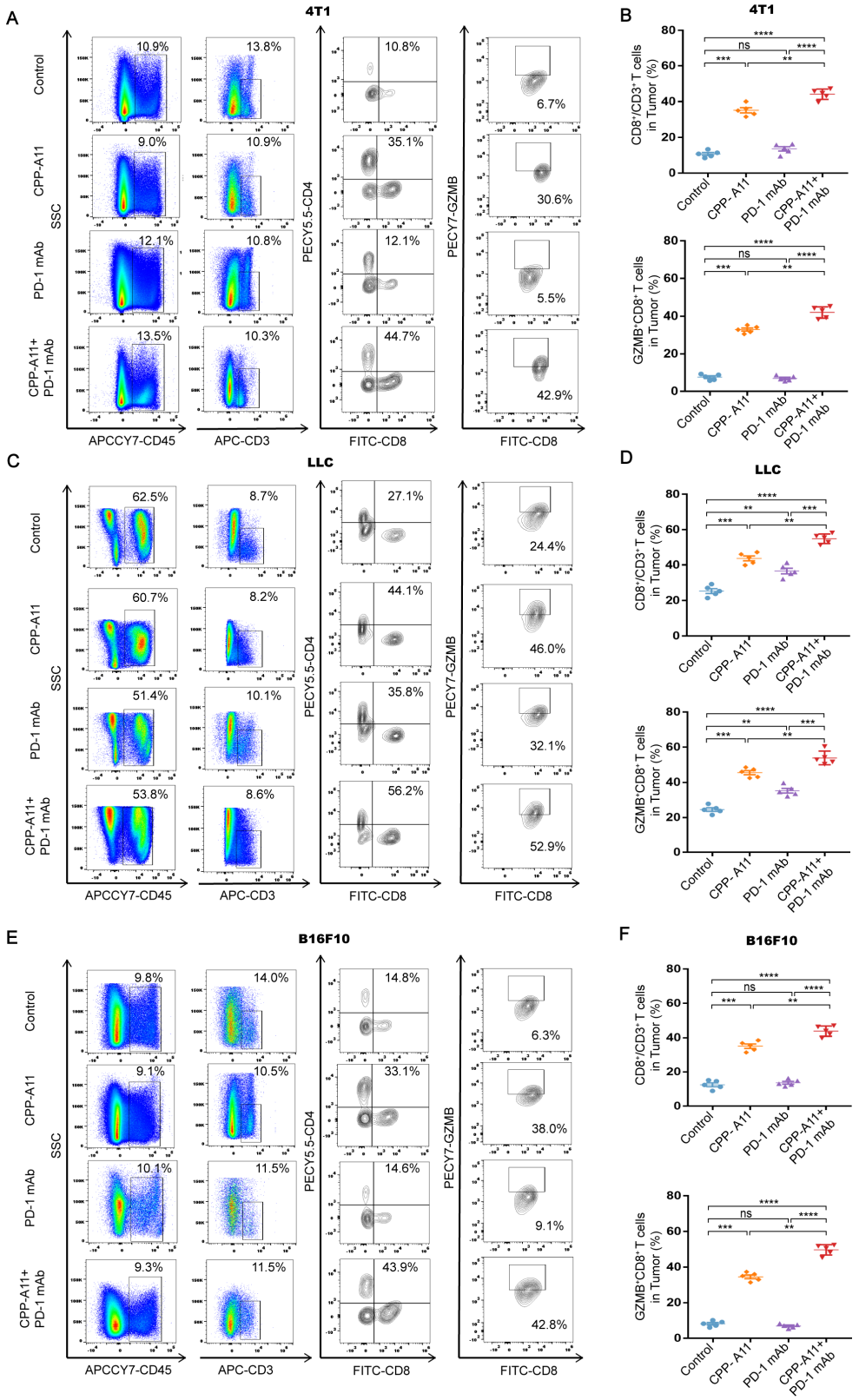

Figure 5 Fluorescence-activated cell sorting (FACS) of tumor infiltrating lymphocytes in the multiple implanted tumors treated with A11 peptide and/or PD-1 mAb. (A–F) The implanted tumors of 4T1, LLC and B16F10 cells were harvested from immune-competent mice received the treatment of CPP-A11 or/and PD-1 mAb, and from their control mice received the peritoneal injection of both CPP and rat IgG2a. Single cell suspension was prepared from each tumor, and $CD45^+$ cells in live cells, $CD3^+$ T cells in $CD45^+$ T cells, $CD8^+$ T cells in $CD3^+$ T cells, and $GZMB^+CD8^+$ T cells in $CD8^+$ T cells were quantified by FACS. Representative FACS results are shown in the figure A, C and E, and the quantitative data are presented in the figure B, D and F. n=5. Data represent mean±SD. Statistical differences were determined by one-way analysis of variance. **P<0.01; ***p<0.001; ****p<0.0001; NS, no significance; PD-1, programmed cell death 1.

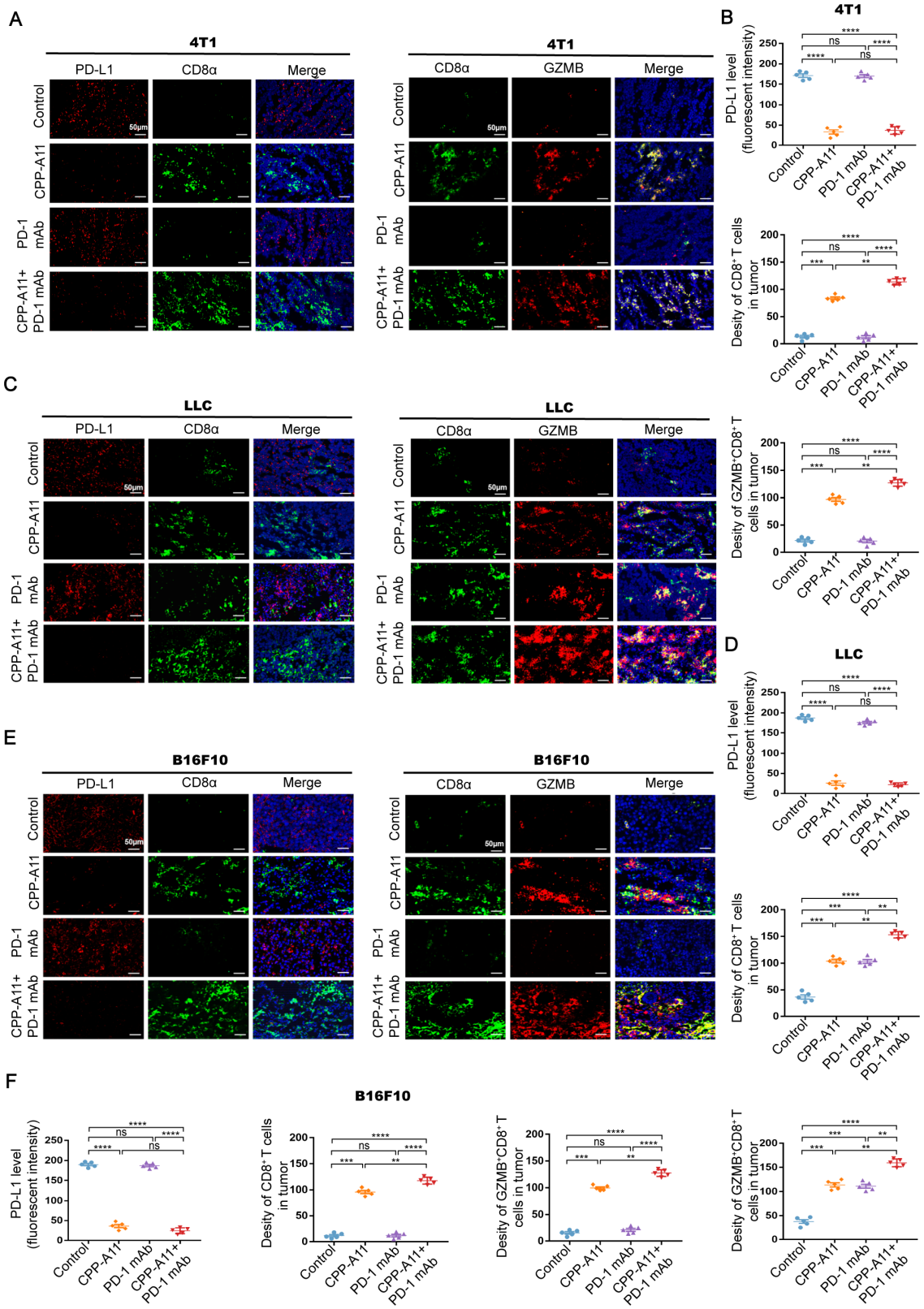

Figure 6 Dichromatic immunofluorescent staining of PD-L1, CD8α and GZMB in the multiple implanted tumors treated with A11 peptide or/and PD-1 mAb. (A–F) The implanted tumors of 4T1, LLC and B16F10 cells were harvested from immune-competent mice received the treatment of CPP-A11 or/and PD-1 mAb, and from their control mice received the peritoneal injection of both CPP and rat IgG2a. Cryostat sections were prepared from each tumor, and expression of PD-L1, CD8α and GZMB in the tumors were detected by dichromatic immunofluorescent staining. Representative images are shown in the figure A, C and E, and the quantitative data are presented in the figure B, D and F. Scale bars=50 µm. n=5. Data represent mean±SD. Statistical differences were determined by one-way analysis of variance. **P<0.01; ***p<0.001; ****p<0.0001; NS, no significance; PD-L1, programmed cell death ligand 1.

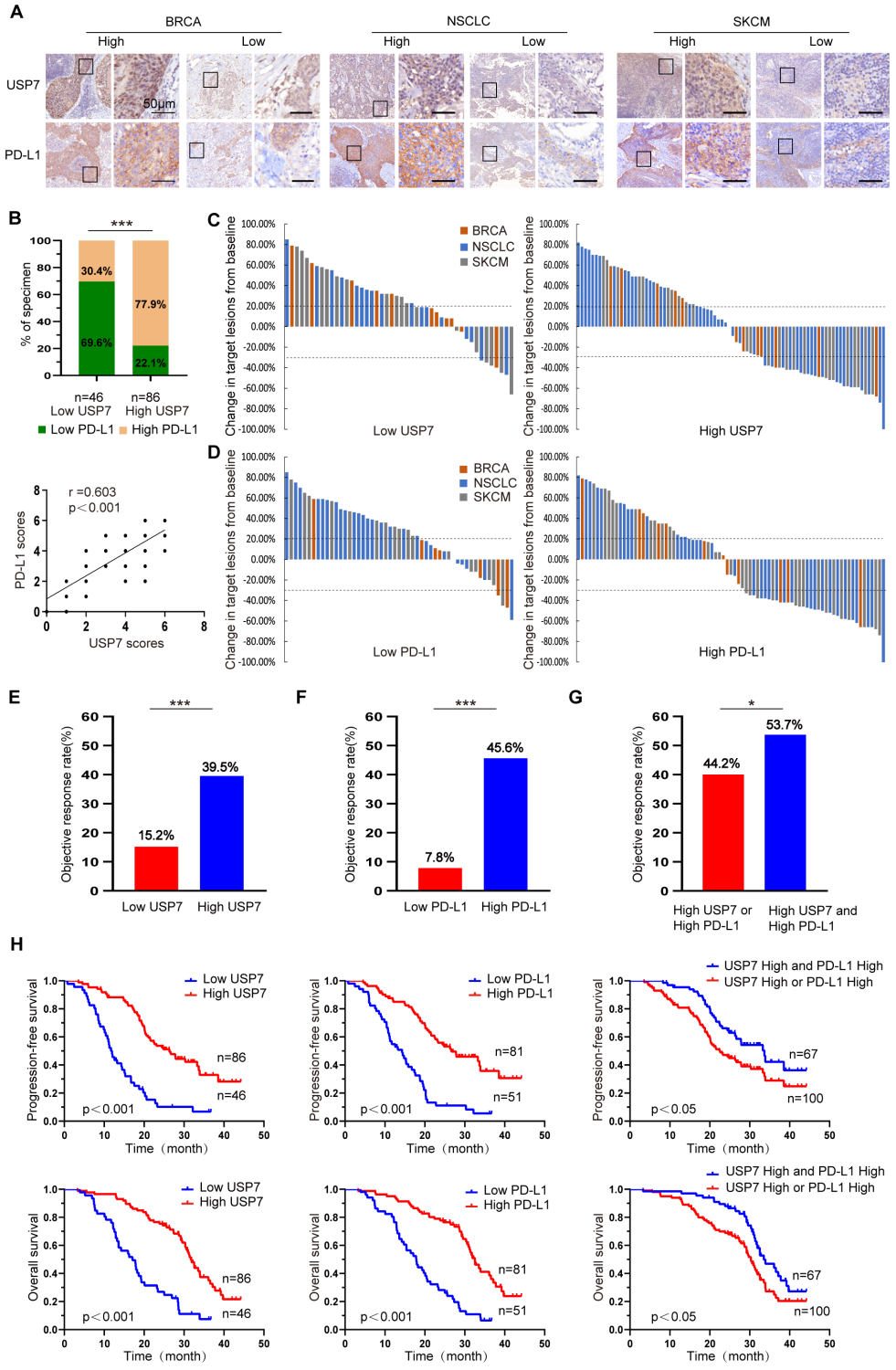

Figure 7 USP7 and PD-L1 expression levels are correlated with efficacy of PD-1 mAb therapy and prognosis in the patients with breast cancer, lung cancer or melanoma. (A and B) Positive correlation between the alterations for USP7 and PD-L1 expression in the breast cancer (BRCA), NSCLC and skin cutaneous melanoma (SKCM) tissues. (A) Representative IHC images of high and low expression of PD-L1 and USP7 in cancer tissues. Scale bars=50 μm. (B) Statistical analysis of correlation between USP7 and PD-L1 expression (p<0.001, Pearson correlation test). (C and D) Waterfall plot showing the best percentage change from baseline in target lesion according to tumor USP7 (C) and PD-L1 (D) expression status. Horizontal dotted lines denote a 30% decrease and 20% increase of tumor size. Best overall response is shown for each patient according to RECIST V.1.1. (E–G) Histograms showing the objective response rate of patients to PD-1 mAb therapy based on the expression levels of USP7 (E), PD-L1 (F) and both the proteins (G). Statistical differences were determined by χ^2 test. *P<0.05; ***p<0.001. (H) Survival analysis of PD-1 mAb-treated patients with breast cancer, NSCLC or SKCM. Kaplan-Meier survival analysis of progression-free survival (*top*) and overall survival (*bottom*) for 132 PD-1 mAb-treated patients based on levels of USP7, PD-L1, or both the proteins. Log-rank test was used to calculate p value. PD-L1, programmed cell death ligand 1.

PD-L1 was significantly higher than that in patients with low levels of USP7/PD-L1 (figure 7E.F). ORR in patients with high levels of both proteins was significantly higher than that in patients with high levels of one protein alone (figure 7G). Survival analysis showed that PD-1 mAb-treated patients with high levels of both USP7 and PD-L1 had a longer progression-free survival (PFS) and overall survival (OS) relative to PD-1 mAb-treated patients with high level of one protein alone (figure 7H). A univariate and multivariate Cox regression analysis showed that a combination of USP7 and PD-L1 was an independent predictor for both PFS (progression-free survival) and OS (online supplemental table S6). Together, these data indicate that a combination of USP7 and PD-L1 could be considered as a marker for predicting efficacy of PD-1 mAb therapy and prognosis in patients with breast cancer, lung cancer or melanoma.

DISCUSSION

ICIs therapy using anti-PD-1/PD-L1 antibody is a main method for treating multiple advanced cancers. However, a significant portion of cancer patients do not benefit from PD-1/PD-L1 mAb therapy.[6] Discovering novel ICIs is required for improving efficacy of ICIs therapy. Recently, several peptides blocking interaction of PD-1 and PD-L1 and enhancing antitumor immunity were discovered.[29–31] Peptides possess many advantages over PD-1/PD-L1 antibody, for example, lack irAEs, have high tumor permeability and low immunogenicity and easily produce.[39 40] In this study, we identified binding of ANXA1-derived peptide A11 with PD-L1, and USP7 as the deubiquitinase of PD-L1, and found that A11 competed with USP7 for binding PD-L1, and degraded PD-L1 by inhibiting USP7-mediated PD-L1 deubiquitination in multiple cancer cells. These findings reveal the role and mechanism of A11-degrading PD-L1 in cancer cells. To the best of our knowledge, it is the first time report that a protein-derived peptide targets PD-L1 degradation by ubiquitin proteasome pathway.

Our previous study has demonstrated that A11 inhibits human nasopharyngeal carcinoma cell growth in immune-deficient mice by targeting EphA2 degradation.[19] Therefore, we first compared the antitumor effect of A11 in immune-deficient and immune-competent mice and observed that antitumor effect of A11 in immune-competent mice was stronger than that in immune-deficient mice, and PD-L1 expression markedly decreased in the implanted tumors of mice received A11 treatment, revealing that an intact immune system enhances A11 antitumor effect, which may be attributed to PD-L1 degradation. Since A11 also targets EphA2 degradation,[19] it may inhibit cancer cell growth in mice partly via targeting EphA2 degradation, indicating that A11 is an antitumor peptide of degrading the two target proteins.

Next, we further evaluated whether the antitumor effect of A11 is involved in tumor immunity and observed that A11 promotes T cell ability of killing cancer cells *in vitro,* and inhibited the implanted tumor growth of multiple cancer cells via suppressing tumor immune escape in mice. We also observed that cotreatment with A11 and PD-1 mAb possessed synergistic antitumor effect via suppressing immune escape, indicating that targeting immune checkpoint with cotreatment of A11 and PD-1 mAb may be a better choice. Moreover, we observed that A11 could inhibit the PD-1 mAb-resistant implanted tumor growth of 4T1 and B16F10 cells, indicating that A11 exerted a better tumor inhibitory effect as compared with PD-1 mAb, the possible reason of which is that A11 degrades PD-L1, whereas PD-1 mAb blocks interaction of PD-1 and PD-L1. Collectively, we cautiously believe that A11 is more efficient than PD-1 mAb in activating anti-tumor immune response.

Although PD-L1 is considered as a ligand of PD-1 that functions on cell surface, several studies have indicated its distribution in other subcellular compartments and redistribution to cell membrane.[41 42] PD-1/PD-L1 mAb efficiently blocks the role of PD-L1 on the cell surface, but its redistribution to cell surface may compromise the efficacy of PD-1/PD-L1 mAb, which highlights the importance of degrading PD-L1 within the whole cell.[43 44] Therefore, A11, which degrades PD-L1 within the whole cell, may be more efficient than PD-1/PD-L1 mAb in suppressing tumor immune escape.

Post-translational modifications of PD-L1 play an important role in regulating PD-L1 protein stability, which directly affects PD-L1-mediated tumor immune escape, and are a promising target for cancer immunotherapy.[45] Recent studies have indicated that several DUBs stabilize PD-L1 by removing its ubiquitination, leading to tumor immunosuppression.[9–11 46] Wang *et al*[47] reported that knockdown of USP7 sensitizes gastric cancer cells to T cell killing. However, whether USP7 is a DUB of PD-L1 in cancer cells needs to be elucidated. In the present study, we demonstrated that USP7 is the DUB of PD-L1 in multiple cancer cells, indicating that USP7 is a potential target for cancer immunotherapy. Therefore, a selective inhibitor of USP7, such as P5091,[48] might have an ability of inhibiting tumor immune escape.

To investigate the clinical significance of USP7 as deubiquitinase of PD-L1, we detected expression levels of USP7 and PD-L1 in tumor tissues from breast cancer, NSCLC and melanoma patients, who received PD-L1 mAb treatment. The results showed that patients with high expression of both USP7 and PD-L1 had better ORR to PD-L1 mAb treatment, and longer PFS and OS relative to patients with high expression of one protein alone, indicating that a combination of USP7 and PD-L1 could be considered as a marker for predicting efficacy of PD-1 mAb treatment in patients with breast cancer, NSCLC or melanoma.

In summary, we discovered the PD-L1 degradation properties of ANXA1-derived peptide A11, which competes with USP7 to bind and degrade PD-L1 in cancer cells. A11 exhibits obvious antitumor effects and synergistic antitumor activity with PD-1 mAb via inhibiting tumor

immune evasion in multiple cancers. A11 targeting PD-L1 degradation represents a potential new strategy for cancer immunotherapy.

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Contributors Z-ZY and Y-YL designed this project, conducted experiments and acquired data. WZ, J-HZ and J-YQ collected patient samples and information and performed experiments. X-PF, LY, DX, TZ, S-SL, DW and QW performed experiments and acquired data. WH, HY and WZ analyzed and interpreted data. Z-QX was responsible for the overall content as guarantor, designed and supervised this project, analyzed and interpreted data and wrote the manuscript.

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