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Original Article

Inhibition of ATR downregulates PD-L1 and sensitizes tumor cells to T cell-mediated killing

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Abstract: The ataxia telangiectasia and Rad3-related (ATR) kinase plays a crucial role in maintaining genome stability in response to DNA damage. Once activated, ATR acts via its downstream target to arrest the cell cycle, promote DNA repair, and enhance cell survival. Therefore, ATR has become an attractive therapeutic target in cancer therapy. Multiple clinical studies have demonstrated that ATR inhibitors can sensitize cancer cells to conventional DNA damaging agents. However, the potential effects of ATR inhibitors on immune response in the tumor microenvironment, especially on the expression of immune checkpoint-related proteins, remain elusive. Here we show that DNA damaging agents, such as ionizing radiation and cisplatin, significantly induce cell surface PD-L1 expression in various cancer cell types. This effect is blocked by depletion or pharmacological inhibition of ATR, suggesting the essential role of ATR in DNA damage-induced PD-L1 expression. Mechanistically, we show that disruption of ATR destabilizes PD-L1 in a proteasome-dependent manner. Furthermore, clinical ATR kinase inhibitor downregulates PD-L1 expression to attenuate PD-L1/PD-1 interaction and sensitize cancer cells to T cell killing. Collectively, our findings indicate that in addition to potentiating DNA damage, ATR inhibitor concurrently downregulates PD-L1 levels and enhances anti-tumor immune responses. Moreover, our data reveal a potential crosstalk between DNA damage response signaling and immune checkpoints, providing a rationale for the combination therapy of ATR inhibitor and immune checkpoint blockade.

Keywords: Cancer biology, DNA damage response, protein kinase, immune checkpoint, protein degradation, ATR, immunotherapy

Introduction

The immune checkpoint signaling axis, programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1), is harnessed by tumor cells to evade the host cytotoxic immune cells in the tumor microenvironment [1]. PD-1 is mainly expressed on activated cytotoxic T cells, whereas its ligand PD-L1 is frequently expressed on tumor cells [2, 3]. The engagement of PD-L1 and PD-1 prevents the activation of cytotoxic T cells, and antibodies against PD-L1 and PD-1 have emerged as promising cancer therapy to enhance the anti-tumor immune response [4]. Although immunotherapy has accomplished durable clinical responses in certain patients, the response rates vary significantly in different tumor types [5]. Therefore, improving our understanding of the intricate regulation of PD-L1 in cancer cells could help to identify biomarkers for immune therapy and provide rationale for combination strategies.

Cells have developed sophisticated strategies to maintain genomic integrity under DNA damage stress. One of the key components of this DNA damage response (DDR) machinery is the ataxia telangiectasia and Rad3-related (ATR)

serine/threonine protein kinase. Once activated by DNA damage, ATR phosphorylates its downstream target Chk1 kinase to induce cell cycle arrest, facilitate DNA repair, and promote cell survival [6]. Therefore, inhibiting ATR is an attractive therapeutic approach for cancer treatment. Recently, highly selective and potent ATR inhibitors, including VE-822 (also known as VX-970) and AZD6738, have been evaluated in early-phase clinical trials either as monotherapy or in combination with a various conventional DNA damaging agents [7]. Indeed, multiple clinical studies demonstrated that inhibition of ATR markedly sensitizes tumor cells, but not normal cells, to irradiation or chemotherapy.

Although there is increasing evidence indicating that DNA damage and repair have a significant impact on the interaction between the tumor and the immune system [8], the effects of ATR inhibitor on anti-tumor immune response, especially on the immune checkpoints, have not been well studied. In this report, we show that ATR inhibitor sensitizes cancer cells to T cell mediated-killing by downregulating cell surface PD-L1 levels and attenuating its interaction with PD-1. Our findings, which reveal an intriguing link between DDR signaling and immune checkpoints, provide new insight into the biological mechanisms underlying PD-L1 regulation in response to DNA damage and highlight the therapeutic implications of the combination of ATR inhibitors with immunotherapy.

Materials and methods

Cell lines and reagents

All cell lines were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. Antibodies against PD-L1, ATR, rH2AX, phospho-ATR (Thr1989) and phospho-CHK1 (Ser345) were purchased from Cell Signaling Technology (Danvers, MA, USA). Actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). VE-821, VE822, and AZD6738 were purchased from Selleck Chemicals (Houston, TX, USA).

Western blotting

Proteins were resolved by polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce Biotechnology, Waltham, MA, USA).

Real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was prepared using the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) according to the manufacture's protocol with 1 μg of total RNA. All RT-PCR reactions were performed in a 20-μl mixture containing 1 × SYBR Green Master Mix (Takara Bio USA, Mountainview, CA, USA), 0.2 μmol/L of each primer, and 2 μl of cDNA template. Primers used are as follows: Human PD-L1: 5'-TGGCATTTGCTGAACGCATTT3' and 5'-TGCAGCCAGGTCTAATTGTTTT-3'. Human glycerradehyde 3-phophate dehydro genase (GAPDH): 5'-GGAGCGAGATCCCTCCAAAAT-3' and 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Real-time PCR was performed using the Applied Biosystem 7500 system (USA) under the following cycling conditions: (step 1) 95°C for 30 sec, (step 2) 40 cycles of 95°C for 5 sec, and 60°C for 34 sec, followed by the melting curve stage. The relative PD-L1 expression level was normalized to that of GAPDH.

Generation of ATR stable knockdown cell lines by lentiviral short hairpin RNA

293T (1.5×10^6) cells were plated in 10-cm dishes. After 24 hours, cells were co-transfected with shRNA constructs (3 μg) together with pCMV-dR8.2 dvpr (3 μg) and pCMV-VSVG (0.3 μg) helper constructs using Lipofectamine 2000 reagent. ShATR and shATM sequences are as follows: shATR #1: AATGTTAGAAGATTAGCGG; shATR #2: TGAAGAACAATTAGTGCCT; shATM: TAAATGACTGTATAGTCAC.

Viral stocks were harvested from the culture medium after two days and then filtered to remove non-adherent 293T cells. To select cells that stably express the shRNA constructs, cells were plated at subconfluent densities and infected with a cocktail of 1 ml of virus-containing medium, 3 ml of regular medium, and 8 μg/ml polybrene, and then selected in 1 μg/ml of puromycin 48 hours after lentivirus infection. About 2 weeks after selection, monolayers of stably infected pooled clones were harvested for use and cryopreserved. ATR stable knockdown efficiency was examined by Western blot analysis.

Flow cytometric analysis of cell surface PD-L1

To evaluate cell surface PD-L1 levels, cells were suspended in 100 uL of cell staining buffer (#420201, BioLegend, San Diego, CA, USA) and incubated with APC-conjugated human PD-L1 antibody (#329708, BioLegend) at room temperature for 30 min. After washing 3 times by centrifugation at 400 *g* for 5 min, stained cells were subjected to fluorescence-activated cell sorting (FACS) analysis using BD FACSCanto II cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo (Tree Star).

Immunofluorescent microscopy

Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 15 min, and then blocked with 5% BSA for 1 hour. After the incubation with primary antibodies overnight at 4°C, cells were then further incubated with the secondary antibodies tagged with fluorescein isothiocyanate, Texas red, or Alexa 647 (Life Technologies) for 1 hr at room temperature, followed by staining nuclei with DAPI contained in the mounting reagent (Invitrogen). HSP90B1 was used as the endoplasmic reticulum (ER) marker. Confocal fluorescence images were captured using a Zeiss LSM 710 laser microscope.

PD-L1 and PD-1 binding assay

To measure PD-1 and PD-L1 protein interaction, cells were incubated with recombinant human PD-1 FC chimera protein (R&D Systems, Minneapolis, MN, USA) at room temperature for 30 min. After washing 3 times by centrifugation at 400 *g* for 5 min, cells were then incubated with human Alexa Fluor 488 dye conjugated antibody (Thermo Fisher Scientific) at room temperature for 30 min. Cells were resuspended in 500 μL o 1 mL of ice cold PBS for FACS analysis. Data were analyzed using FlowJo (Tree Star).

T-cell killing assay

MDA-MB-231 cells were seeded in a 24-well plate. Human peripheral blood mononuclear cells (STEMCELL, Vancouver, BC, Canada)) were activated with 100 ng/mL CD3 antibody, 100 ng/mL CD28 antibody, and 10 ng/mL IL2 (#31-7303; #302913; #589102, BioLegend) and then cocultured with MDA-MB-231cells at 10:1 ratio. After 96 hours, cells were fixed with methanol followed by crystal violet staining.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. All data are presented as mean ± standard deviation (s.d.). Student's t-test was used to compare two groups A p value < 0.05 is considered statistically significant.

Results

Ionizing radiation (IR)/cisplatin-induced DNA damage upregulates cell surface PD-L1 in various cancer cell types

To test the effect of DNA damage on cell surface PD-L1 levels, various cancer cell lines were treated with IR (10 Gy) and their cell surface PD-L1 levels were analyzed by flow cytometry. PD-L1 levels on cell surface were significantly higher after IR treatment for 24 hours in MDA-MB-231 (231) breast cancer cells, A549 lung cancer cells and HeLa cervical cancer cells compared with the untreated cells (**Figure 1A**). To further validate the effect of DNA damage, we treated the cells with cisplatin (CDDP, 10 uM), another DNA damaging agent and a widely used chemotherapy drug, and then measured PD-L1 levels by flow cytometry. Similar to IR, PD-L1 levels also increased in cells treated with cisplatin (24 hours; **Figure 1B**), and this PD-L1 induction was sustained for at least 48 hours (Supplementary Figure 1). We also observed significant induction of PD-L1 total protein levels after IR or cisplatin treatment

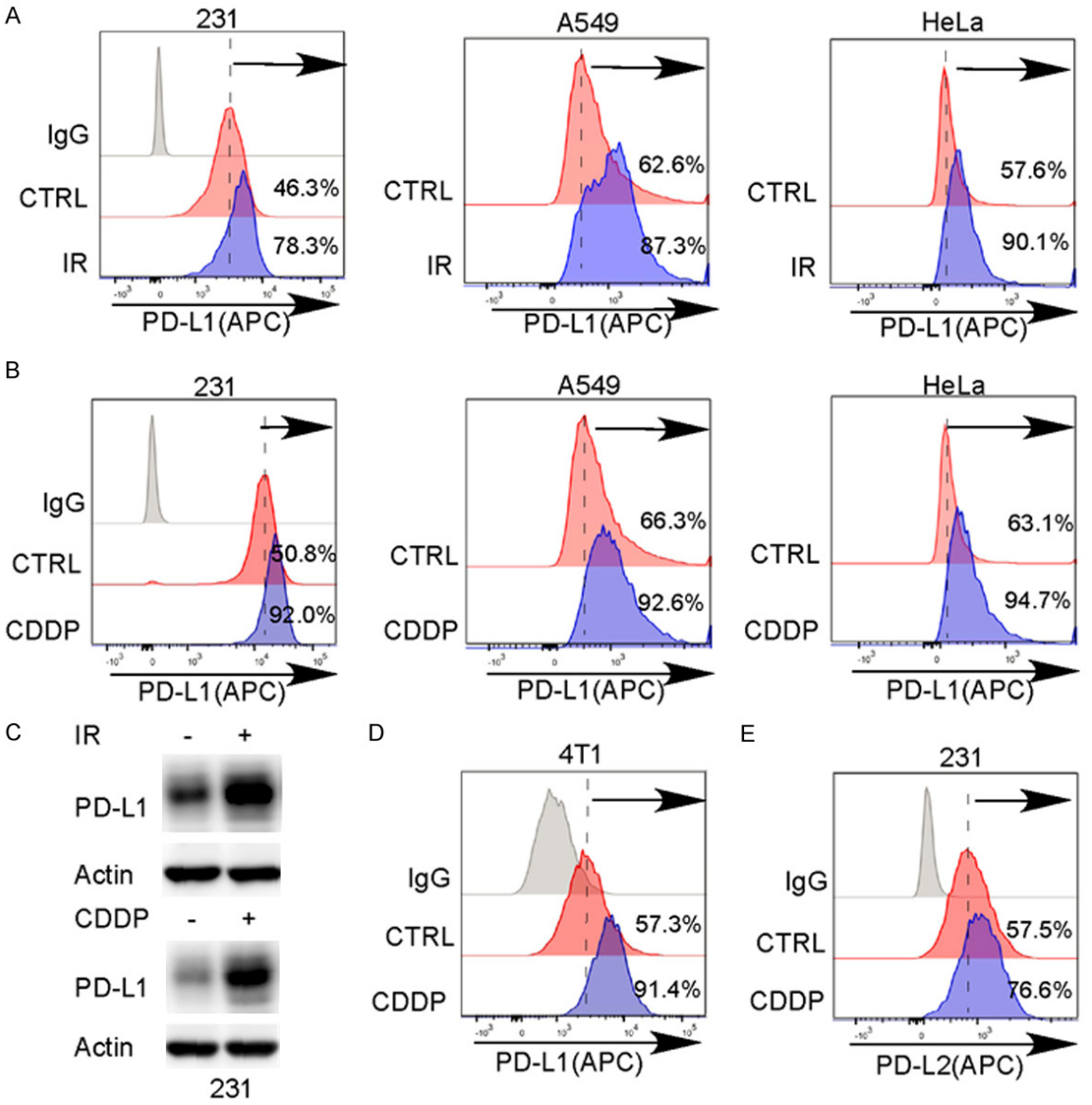
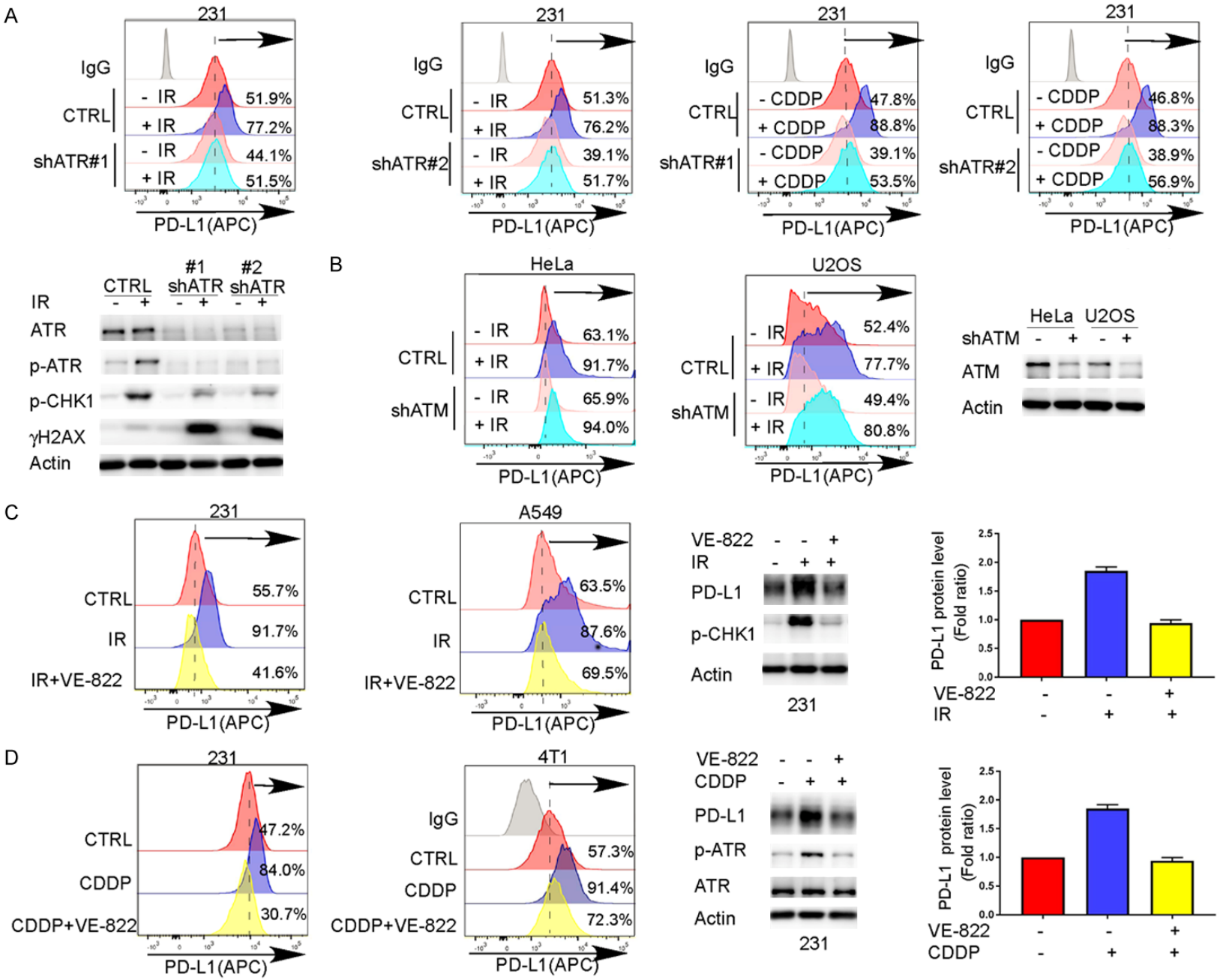


Figure 1. IR/cisplatin-induced DNA damage upregulates cell surface PD-L1 in various cancer cell lines. A. MDA-MB-231, A549 and HeLa cancer cell lines were treated with IR (10 Gy) for 24 hours and their cell surface PD-L1 levels were analyzed by flow cytometry. B. MDA-MB-231, A549 and HeLa cancer cell lines were treated with cisplatin (10 μM) for 24 hours and their cell surface PD-L1 levels were analyzed by flow cytometry. C. MDA-MB-231 cells were treated with IR (10 Gy) or cisplatin (10 μM) for 24 hours, and total PD-L1 protein levels were evaluated by Western blotting. D. Mouse 4T1 mammary tumor cells were treated with cisplatin (10 μM) for 24 hours and cell surface PD-L1 levels were analyzed by flow cytometry. E. MDA-MB-231 cells were treated with cisplatin (10 uM) for 24 hours and cell surface PD-L2 levels were analyzed by flow cytometry.

(**Figure 1C**). In addition to human cancer cells, DNA damage also enhanced PD-L1 levels in mouse 4T1 mammary tumor cells (**Figure 1D**). Interestingly, we also observed enhanced the levels of another PD-l ligand, PD-L2, after cisplatin treatment (**Figure 1E**). Together, these results indicated that both IR- and cisplatin-induced DNA damage upregulates immune checkpoint proteins.

DNA damage-induced PD-L1 requires ATR

Next, we sought to identify the key kinase that mediates DNA damage-induced PD-L1. Since ATR is one of central kinases activated by DNA damage, we asked whether ATR is involved in the process. We generated ATR-knockdown 231 stable cell line by two ATR-specific shRNAs (knockdown efficiency was confirmed by Wes-


A
231
IgG
CTRL
- IR
+ IR
shATR#1
shATR#2
51.9%
77.2%
44.1%
51.5%
51.3%
76.2%
39.1%
51.7%
- CDDP
+ CDDP
47.8%
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39.1%
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46.8%
88.3%
38.9%
56.9%
PD-L1(APC)
CTRL
#1 shATR
#2 shATR
IR
ATR
p-ATR
p-CHK1
γH2AX
Actin
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HeLa
U2OS
63.1%
91.7%
65.9%
94.0%
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A549
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PD-L1
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D
4T1
47.2%
84.0%
30.7%
57.3%
91.4%
72.3%
CDDP
CDDP+VE-822
p-ATR
ATR

Figure 2. ATR depletion or inhibition attenuates DNA damage-induced PD-L1 upregulation. A. MDA-MB-231-pGIPZ and MDA-MB-231 shATR cells were treated with IR (10 Gy) or cisplatin (10 μM) for 24 hours followed by flow cytometric analysis of cell surface PD-L1 levels. ATR, p-ATR, p-CHK-1 and rH2AX levels were analyzed by Western blotting. B. HeLa-pGIPZ and Hela-shATM cells, and U2OS-pGIPZ and shATM cells were treated with IR (10 Gy) for 24 hours followed by flow cytometric analysis of cell surface PD-L1 levels. ATM protein levels were analyzed by Western blotting. C. MDA-MB-231 and A549 cells were treated with IR (10 Gy) for 24 hours with or without VE-822 (0.1 uM) pretreatment followed by flow cytometric analysis of cell surface PD-L1 levels. PD-L1 and p-CHK1 levels were analyzed by Western blotting. Relative fold ratio of PD-L1 protein levels was shown. D. MDA-MB-231 and 4T1 cells were treated with cisplatin (10 μM) for 24 hours with or without VE-822 (0.1 μM) pretreatment followed by flow cytometric analysis of cell surface PD-L1 levels. PD-L1, ATR, p-ATR levels were analyzed by Western blotting. Relative fold ratio of PD-L1 protein levels is shown.

tern blot, **Figure 2A**), and the cells were subsequently treated with IR (10 Gy) followed by flow cytometric analysis of cell surface PD-L1 levels. As shown in **Figure 2A**, compared with the parental cells, ATR depletion significantly attenuated IR-induced PD-L1, suggesting that ATR kinase plays a key role in DNA damage induced PD-L1 upregulation. We also observed a concomitant decrease in the phosphorylation of ATR and its key substrate CHK-1, and an increase in DNA damage accumulation marker rH2AX. To further validate these results, we treated ATR-knockdown cells with cisplatin (10 μM) and evaluated the cell surface PD-L1 levels. Similarly, ATR knockdown also substantially abrogated cisplatin-induced PD-L1 upregulation (**Figure 2A** and Supplementary Figure 2A). Ataxia telangiectasia mutated (ATM) is another major protein kinases activated by DNA damage. Next, we asked whether ATM is involved in DNA damage-induced PD-L1 upregulation. For this experiment, we utilized HeLa and U2OS osteosarcoma cells, but not 231, because 231 cells are deficient of ATM. Depletion of ATM had no apparent effects on IR-induced PD-L1 upregulation (**Figure 2B**). These findings indicated that ATM is not involved in the process. Together, these results demonstrated that DNA damage-induced PD-L1 requires ATR but not ATM.

To investigate whether inhibition of ATR activity can attenuate DNA damage-induced PD-L1, we treated cells with IR in the absence or presence of ATR inhibitor VE-822 (0.1 uM). As shown in **Figure 2C**, IR-induced ATR activation was substantially inhibited by VE-822 pretreatment, as indicated by the decrease in the phosphorylation of CHK-1. VE-822 attenuated IR-induced PD-L1 upregulation in various cancer cell lines, including 231, A549, HeLa and U2OS (**Figure 2C** and Supplementary Figure 2B). We observed similar results in 231 and 4T1 cells treated with cisplatin (**Figure 2D**). Moreover, cisplatin-induced PD-L2 was also blocked by VE-822 (Supplementary Figure 2C). Taken together, these results demonstrated that ATR depletion or inhibition significantly decreases DNA damage-induced PD-L1 and PD-L2, indicating the essential role of ATR in regulating immune checkpoint proteins in response to DNA damage.

ATR inhibitor sensitizes cancer cells to T cell killing by destabilizing PD-L1 and attenuating PD-L1/PD-1 interaction

Because PD-L1 is constitutively expressed in 231 cells, we sought to examine whether endogenous PD-L1 is affected by inhibition of ATR. As shown in **Figure 3A**, VE-822 treatment led to a marked decrease in endogenous PD-L1 protein levels. We also observed similar downregulation of cell surface PD-L1 in the presence of VE-822 by flow cytometry (**Figure 3A**). Additionally, downregulation of total endogenous PD-L1 protein levels occurred in a dose-dependent manner for two other ATR inhibitors (AZD6738 and VE-821) tested (Supplementary Figure 3A). Next, to determine whether ATR mediated PD-1 regulation occurs at the transcriptional or post-transcriptional level, we first examined PD-L1 mRNA levels in 231 cells treated with or without VE-822. Compared with the untreated cells, VE-822 induced a mild downregulation of PD-L1 mRNA levels (**Figure 3B**), which cannot account for the more than 3-fold reduction of PD-L1 protein, suggesting that the significant downregulation of PD-L1 protein by inhibition of ATR may be attributed to additional post-translational mechanisms. To address this, we examined the effects of the proteasome inhibitor MG132 on ATR-mediated regulation of PD-L1. Treatment with VE-822 caused a marked reduction in PD-L1 protein level, which was blocked by the addition of MG132 (**Figure 3B**). Similar results were observed by flow cytometry (**Figure 3B**). These results indicate that ATR inhibitor destabilizes PD-L1 protein in a proteasome-dependent manner.

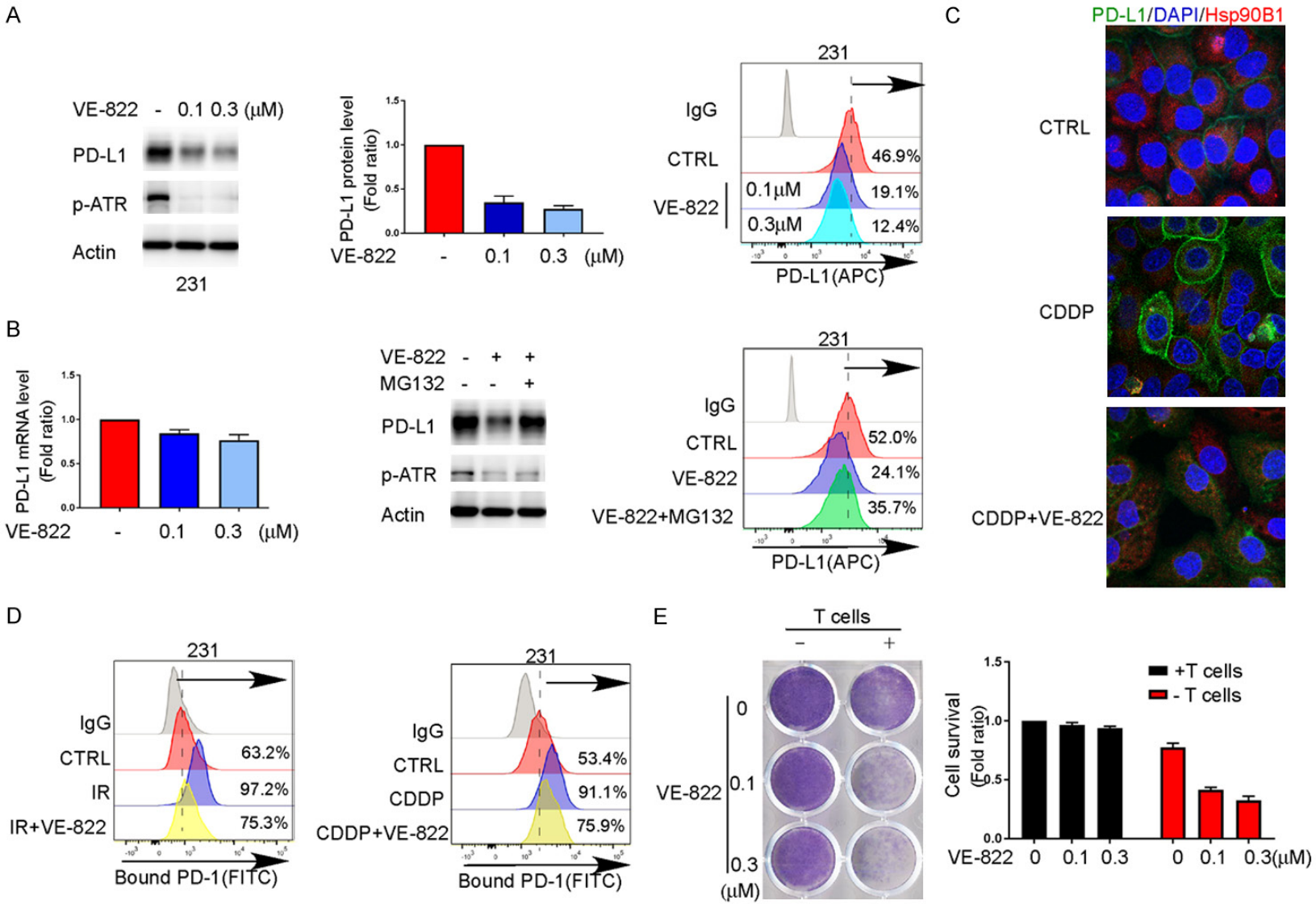

Figure 3. Inhibition of ATR attenuates PD-L1/PD-1 interaction and sensitizes cancer cells to T cell killing. A. MDA-MB-231 cells were treated with VE-822 at indicated concentrations for 24 hours, total PD-L1 protein levels were evaluated by Western blotting and cell surface PD-L1 levels analyzed by flow cytometry. Relative fold ratio of PD-L1 protein levels is shown. B. MDA-MB-231 cells were treated with the indicated concentrations of VE-822 for 24 hours. PD-L1 mRNA levels were measured by RT-PCR. MG132 was added 6 hours before collecting the cells for flow cytometry or Western blotting. Relative fold ratio of PD-L1 mRNA levels is shown. C. HeLa-GFP-PD-L1 cells were treated with cisplatin (10 μM) for 24 hours with or without VE-822 (0.1 μM) pretreatment, and PD-L1 localization was analyzed by

immunofluorescence. D. MDA-MB-231 cells were treated with IR (10 Gy) or cisplatin (10 μM) for 24 hours with or without VE-822 (0.1 uM) pretreatment, and the interaction between PD-L1 and PD-1 was analyzed by flow cytometry. E. MDA-MB-231 cells were treated with the indicated concentrations of VE-822 and co-cultured with or without activated T cells for 3 days followed by crystal violet staining to evaluate the cancer cell survival. Relative fold ratio of surviving cell intensities is shown.

To investigate the functional significance of our findings, we first validated the downregulation of cell surface PD-L1 by ATR inhibitor using immunofluorescence (**Figure 3C**) and then evaluated the interaction between PD-L1 and PD-1 in cells treated with or without ATR inhibitor by flow cytometry. As shown in **Figure 3D**, both IR and cisplatin enhanced the binding of PD-1 to PD-L1, and this induction was markedly attenuated by VE-822 pretreatment, indicating that ATR inhibitor downregulates membranous PD-L1 and reduces its interaction with PD-1. Given that the binding of PD-L1 with PD-1 can suppress T cell activation, we asked whether ATR inhibition affects T cell-mediated cancer cell killing by co-incubating 231 cells with activated T cells in an *in vitro* assay. As shown in **Figure 3E**, ATR inhibitor sensitized cancer cells to T-cell killing. These results demonstrated that inhibition of ATR sensitize cancer cells to T cell killing by destabilizing PD-L1 and attenuating the PD-L1/PD-1 interaction.

Discussion

Genomic destabilizers, such as radiation and chemotherapy, are used for cancer treatment to induce lethal DNA damage in tumor cells. Although those conventional DNA damaging therapeutics are known to be immunosuppressive, the potential effects on immune checkpoints remain unclear. Recent studies indicated that the combination of IR and anti-PD-L1 treatment synergistically promotes anti-tumor immunity in a mouse model [9]. Anti-PD-L1 therapy also demonstrated beneficial effects when used in combination with cisplatin [10]. Consistent with the previous reports, we showed in the current study that IR or cisplatin induced cell surface PD-L1 levels in various cancer lines and provided a rationale for the combination of anti-PD-L1 and conventional DNA damaging agents. Furthermore, we identified an essential role of the ATR kinase in DNA damage-induced PD-L1 upregulation, thus providing novel molecular insights into the role of ATR kinase in regulating immune checkpoints. In line with our study, PD-L1 was recently reported to be regulated by DNA repair-related proteins [11].

Immunotherapy has emerged as powerful weapons in the clinical oncology, but PD-L1 inhibitors are not effective across all cancer types. Multifactorial biomarkers, including tumor mutational load, infiltrating CD8+T cell intensity, and PD-L1 expression levels, have been proposed as distinct biomarkers of response to anti-PD-L1 therapies [12]. Genomic instability induced by disruption of normal DNA repair function could lead to increased tumor mutational load. Thus, targeting DDR enzymes may be a useful therapeutic strategy. There are multiple studies on the combination of immune checkpoint and DDR inhibitors, such as those targeting PARP and ATR. We previously reported that PARP inhibition induces immunosuppressive effects by upregulating PD-L1 [13], which further support the use of the combination of anti-PD-L1 and PARP inhibitor. In the current study, we demonstrated that in addition to potentiating DNA damage induced by conventional cancer therapy (Supplementary Figure 3B), ATR inhibitor concurrently sensitized cancer cells to T-cell mediated killing by downregulating PD-L1 levels and decreasing PD-1 and PD-L1 interaction. Notably, ATR inhibitor did not directly affect T cell receptor activation (Supplementary Figure 3C). Based on our findings, it is reasonable to speculate that combining ATR inhibitor, which downregulate PD-L1 expression, with other types of immunotherapy, such as anti-CTLA-4 [14] or anti-TIM-3, may lead to increased therapeutic efficacy.

ATR plays a critical role in the activation of the G2/M cell cycle checkpoint [15], which prevents cells with damaged DNA from entering mitosis. Our findings that inhibition of ATR, the central transducer of checkpoint signaling, downregulated the levels of the immune checkpoint molecule PD-L1 in multiple cancer cell lines, shed new lights on the intriguing crosstalk between the two checkpoints: cell cycle checkpoint and immune checkpoint. Consistent with our findings, a recent study showed that PD-L1 protein levels fluctuated during cell cycle progression and peaked in the G2 and M phases [16]. Therefore, it appears that cancer cells acquire several strategies to survive under DNA damage stress, including the activation of the

cell cycle checkpoints to prevent the premature entry into mitosis and the induction of immune checkpoint to avoid immune surveillance and escape from host immune attacks by the cytotoxic T cells.

PD-L1 can be regulated both at the transcriptional level and post-translational levels. Several post-translational modifications (PTMs) of PD-L1, including phosphorylation [17], glycosylation [17, 18] and ubiquitination [16, 19], have been reported to regulate PD-L1 stability. In the current study, we showed that ATR inhibition mainly destabilized PD-L1 in a proteasome-dependent manner, suggesting that post-translational regulation may play a major role in PD-L1 regulation by ATR. It remains interesting to further investigate the molecular mechanism underlying ATR-mediated PD-L1 regulation.

In summary, inhibition of ATR sensitizes cancer cells to T-cell mediated-killing by downregulating cell surface PD-L1 levels, which limits its interaction with PD-1. Our findings revealed an intriguing link between DDR signaling and immune checkpoints and have important therapeutic implications for the combination of ATR inhibitors and immunotherapy.

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Disclosure of conflict of interest

None.

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Supporting Information

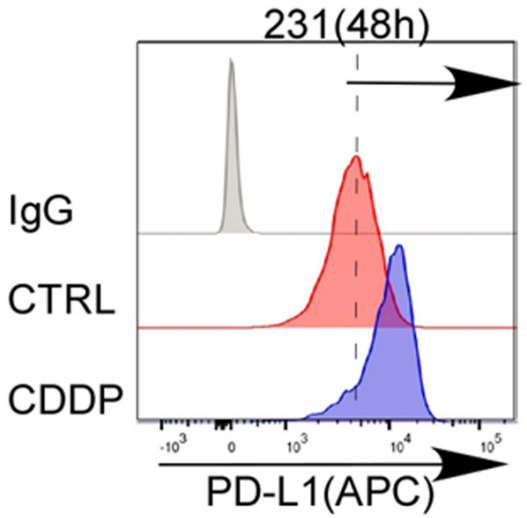
Supplementary experimental procedures

Crystal violet assay

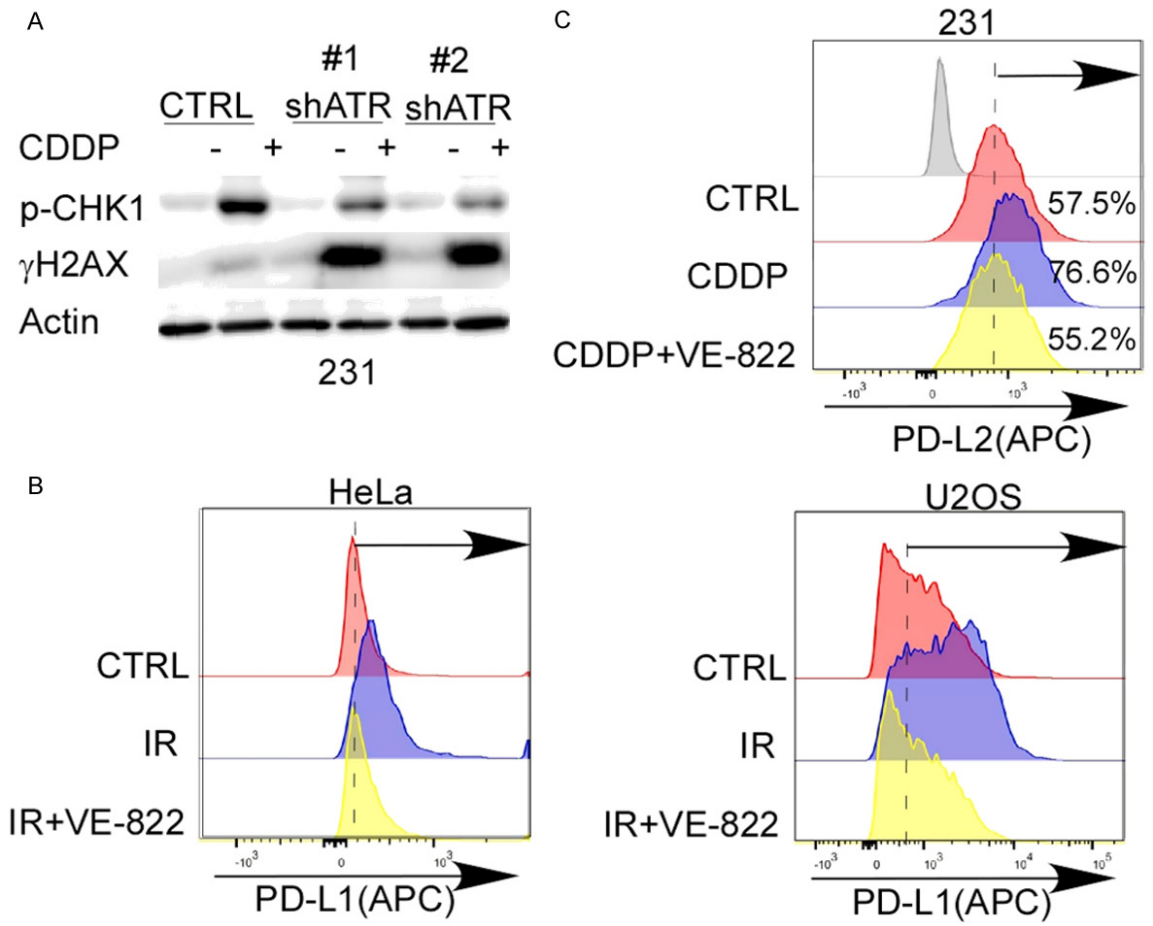
Cells were seeded in a 24-well plate treated with or without cisplatin at the indicated concentrations. After incubation at 37°C for 2 days, cell were fixed with methanol at -20°C for 20 min, and stained with 0.05% crystal violet at room temperature for an additional 1 hour.

NFAT luciferase reporter assay

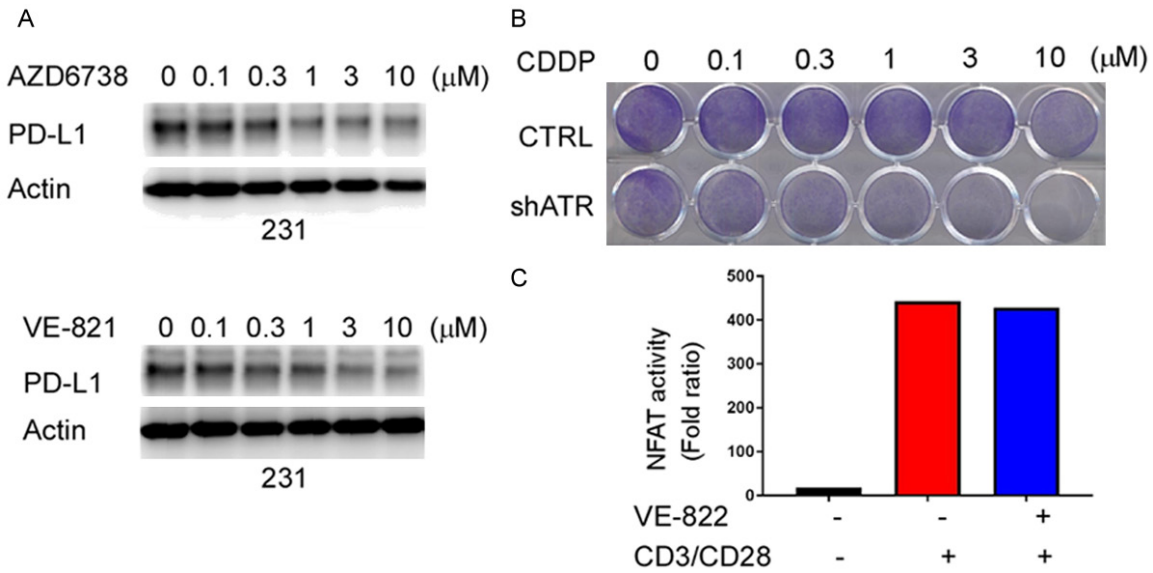
Jurkat T cells were transfected with reporter constructs, pNFAT-Luc and TK-Renilla. After 24 hours, cells were stimulated with anti-CD3/CD28 with or without VE-822. Cell lysates were then prepared and Luciferase activity was measured with Dual Luciferase assay kit (Promega, Madison, WI) by TD20/20 luminometer (Turner Designs Inc, Sunnyvale, CA) as instructed by the manufacturer. Normalized luciferase activity was presented as the ratio of firefly luciferase activity to Renilla luciferase activity.



Supplementary Figure 1. MDA-MB-231 cells were treated with cisplatin (10 μM) for 48 hours, and cell surface PD-L1 levels were analyzed by flow cytometry.



Supplementary Figure 2. A. MDA-MB-231-pGIPZ and MDA-MB-231 shATR cells were treated with cisplatin (10 μM) for 24 hours, and p-CHK-1 and rH2AX levels were analyzed by Western blotting. B. HeLa and U2OS cells were treated with IR (10 Gy) for 24 hours with or without VE-822 (0.1 μM) pretreatment followed by flow cytometric analysis of cell surface PD-L1 levels. C. 231 cells were treated with cisplatin for 24 hours with or without VE-822 (0.1 μM) pretreatment followed by flow cytometric analysis of cell surface PD-L2 levels.



Supplementary Figure 3. A. MDA-MB-231 cancer cells were treated with AZD6738 or VE-821 at indicated concentrations for 24 hours. Total PD-L1 protein levels were evaluated by Western blotting. B. MDA-MB-231-pGIPZ and MDA-MB-231-shATR cells were treated with the indicated concentrations of cisplatin for 48 hours, followed by crystal violet staining to evaluate the cancer cell survival. C. Jurkat T cells were transiently transfected with NFAT luciferase report plasmid followed by CD3/CD28 activation in the absence or presence of VE-822, and relative fold ratio of NFAT activity was analyzed and shown.