# Interference with pathways activated by topoisomerase inhibition alters the surface expression of PD-L1 and MHC I in colon cancer cells

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**Abstract.** Topoisomerase inhibitors are clinically used to treat various cancer types, including colorectal cancer. These drugs also activate signaling pathways that modulate cell survival and immune cell functions. Immunotherapy is promising for certain tumors, including microsatellite instable colorectal cancer, but not for microsatellite stable colorectal cancer. The reasons for this lack of responsiveness are largely unknown. Understanding how colorectal cancer cell-surface proteins interact with tumor-resident immune cells may offer an opportunity to identify molecules that, if targeted, may render tumor cells visible to immune cells. The present study used flow cytometry, fluorescent staining and immunoblotting to examine if inhibition of pathways activated by topoisomerase-targeting drugs may modulate the outcomes of treatment through effects on cell cycle arrest and apoptosis, and by altering surface expression levels of programmed death-ligand 1 (PD-L1) or major histocompatibility complex protein I (MHC I). Inhibition of either NF-κB or DNA-damage response (DDR) potently enhanced cell death in combination with topoisomerase inhibition, while only NF-κB inhibition increased MHC I. PD-L1 upregulation was moderately affected by NF-κB or DDR inhibitors, while both topoisomerase inhibitors and DNA damaging agents may enhance the surface expression of MHC I molecules on colon cancer cells. Such enhanced expression of MHC I may be suppressed by inhibitors of ataxia-telangiectasia mutated or checkpoint kinase kinases. Additionally, adaptive tolerance to topoisomerase inhibition caused altered cell cycle response, and reduced the expression levels of both PD-L1 and MHC I on both microsatellite instable and stable colon cancer cell lines. Therefore, targeted modulation of DDR pathways, PD-L1, MHC I or other immune regulators in colon cancer cells may make them more visible to immune cells and enable rational combination of conventional therapy with immunotherapy.

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## Introduction

Immunotherapy has improved the options for cancer therapy by enabling the immune cells to target cancer cells in a patient. Immunotherapy is now clinically offered for melanoma and lung cancer patients, while some cancers, including the majority of colorectal cancer (CRC), remain recalcitrant (1-3).

Immunotherapy has been approved for microsatellite instable (MSI) types of CRC (4), which account for only 10-15% of the total CRC cases. This leaves up to 85% of the cases, most of which display microsatellite stability (MSS), to be managed through the conventional approach. While early and localized cases of CRC may be surgically resected with or without neoadjuvant therapy, advanced and metastatic cases are treated with chemotherapy drugs or combinations of surgery, radiation and drugs (2,5).

Studies on MSI CRC responsive to immunotherapy suggest that the genomic instability in such tumors may enable generation of neoantigens that are recognized by immune cells (4,6,7). Such instability combined with defects in mismatch repair would enhance the visibility of altered cancer cells to T-cells which could orchestrate cytotoxic responses. Despite such mechanistically sound explanation for the visibility of MSI tumors, not all MSI tumors respond to immunotherapy, and the reasons for this remain to be uncovered.

Multiple theories have been proposed for the non-responsiveness of most CRC tumors to immunotherapy (6). One of the possibilities is immune-exclusion, where the lack of immune cells in the microenvironment may be the primary factor. However, the abundance of immune cells in the microenvironment has been associated with either positive or negative outcomes (5,8-10). In addition to the physical presence of immune cells in the tumor, other factors including the sub-types and proportions of the immune cells, their activation status, and the cytokine microenvironment may modulate the effector functions and clinical outcomes (6,11,12).

Cancer cells are known to respond to replication stress, chemotherapy, and radiation by activating DNA-damage response (DDR) and nuclear factor-κB (NF-κB) pathways (13-18), both of which may serve as survival mechanisms for the cells damaged during therapy. Studies on CRC treatment to strategically combine chemotherapy, radiation, and immunotherapy are ongoing, with some encouraging preliminary results (19-26). However, mechanistic studies on resistance mechanisms, and experiments to rationalize combination strategies are urgently needed (27). Combination therapies could markedly benefit from studies that identify effective strategies for patient selection, drugs to combine, and timing and sequence of combination.

In this study, we examined the responses of CRC cell lines treated with clinically used topoisomerase inhibitor in combination with drugs that interfere with pathways initiated by topoisomerase inhibition. We show that the cell surface expression of immunoregulatory molecules on cancer cells may be modulated by the combination of topoisomerase inhibitors and DDR pathway blockers. This suggests that chemotherapy-activated pathways may be identified and selectively targeted to enable the broader clinical use of immunotherapy in CRC and to improve therapeutic outcomes.

## Materials and methods

*Cell lines and culture*. Colon cancer cells (SW620, RKO, HCT116) and the colorectal cancer cell line (HT-29) were purchased from the American Type Cell Culture (ATCC) collection. No further authentication was done. Soon after receipt, multiple stock cultures were prepared and stored in liquid nitrogen, and periodically thawed and used for specific experiments. All parental cell lines were grown in DMEM (Dulbecco's Modified Eagle's Medium, Corning #10-013-CV) culture medium containing 10% fetal bovine serum plus 10 μg/ml ciprofloxacin. SN-38-tolerant cell lines were developed as previously described (28) using culture medium containing 10% fetal bovine serum, penicillin (10,000 U/ml), and streptomycin (10 mg/ml). Briefly, parental cells were exposed to an initial SN-38 dose of 1 nM and cultured to a confluency of 80% for three passages (~6 weeks). The cells that survived the initial SN-38 treatment were then exposed to 5 nM SN-38 for three passages (~8 weeks) and to 10 nM for three more passages (~eight weeks). Finally, the SN-38 concentration was increased to the clinically relevant plasma drug concentration of 50 nM for three weeks.

*Drugs and reagents*. Stock concentrations of the compounds were prepared in Dimethyl sulfoxide (DMSO) and stored at -20˚C. SM-7368 (481411-5mg), CPT (C9911-250MG), KU60019 (SML1416-5MG), Irinotecan (IRI) (SKU-I1406-50MG), and VE-822 (1232416-25-9) were purchased from Sigma-Aldrich (St. Louis, MO). AZD7762 (Enzo, ENZ-CHM185-0005) from ENZO. Flow Cytometry Staining Buffer (1X) (Cat# FC001), Mouse PE-IgG2a (R&D IC003p), and Human HLA Class I (MHC I) Phycoerythrin mAb (Clone W6/32) were purchased from R&D Systems. Unless indicated otherwise, the drugs were used at the following concentrations: CPT 0.5-1 μM; SM-7368 5 μM; irinotecan 25 μg/ml; VP-16 (Etoposide, LC laboratories) 25 μM; γ-IFN (Sigma) 50 ng/ml; VE822 (Selleckchem) 100 nM; AZD7762 100 nM, KU60019 at 1 μM; erlotinib (LC laboratories) 20 μM; oxaliplatin (Sigma) 10 μM; taxol (Sigma) 50 nM; 5-Fluorouracil (5-FU, Sigma) 10 μM. For experiments in this study, single drug concentrations were pre-determined based on the length of treatment (24-48 h) that induced the expression of target proteins without visibly killing the treated cells.

*Immunoblotting*. Monolayer culture cells were treated in 6-cm culture dishes and lysed in RIPA buffer containing a cocktail of protease and phosphatase inhibitors. Samples containing equivalent protein concentrations were resolved by SDS-PAGE and analyzed by immunoblotting using pre-made 4-15% gradient gels. AI680 digital imager (GE Lifesciences, Pittsburg, PA, USA) was used to scan the chemiluminescent signals. Primary antibodies, at 1:1,000 dilutions, used to target PD-L1 (catalog #13684, rabbit mAb) and were obtained from Cell Signaling Technologies (Danvers, MA, USA). Peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were from Millipore (Temecula, CA, USA) and used at 1:3,000 dilutions.

*MHC-I and PD-L1 detection by fluorescence activated cell sorting (FACS)*. Colon cancer cells (HCT116, RKO, SW620), colorectal HT-29 cells and the SN-38-adapted derivatives were seeded and treated with drugs (Irinotecan, VE822, KU60019, VE822, AZD) in 6-well plates for 24 h. Later, the culture medium was removed by aspiration, and cells were harvested by brief trypsinization and neutralized by adding 1 ml of medium for each well. The cells were collected in a 2 ml tube on ice and centrifuged for 2 min at 3K rpm. The medium was removed by aspiration, and the pellet was washed two times in 1 ml cold staining buffer (FC001) (centrifuge for 2 min at 3,000 rpm). Each pellet of about $10^6$ cells was resuspended in 100 μl staining buffer with the respective antibody (100 μl X no. of tubes for Isotype control (Mouse PE-IgG2a (IC003p) & 100 μl X no. of tubes for HLA I (MHC I) Ab (FAB7098P PE conjugated anti-human HLA Class I). Then, the cells were incubated for 1 h on ice in the dark and washed two times with 1 ml staining buffer. The final pellet was resuspended with 500 μl staining buffer and filtered (70 microns filter) into FACS tubes (on ice) and analyzed. For PD-L1 FACS, each pellet of about $10^6$ cells was resuspend in 100 μl staining buffer with the respective antibody (100 μl with 2.5 μl Isotype Ab R&D (AF488) & 100 μl with 2.5 μl PD-L1 Ab R&D (AF488) and incubated for 1 h on ice in the dark and washed two times with 1 ml staining buffer. The final pellet was resuspended in 500 μl staining buffer and filtered (70 Microns filter) into FACS tubes (on ice) before data collection on BD FACSCalibur, (Becton Dickinson Biosciences, San Jose, CA, USA) using FlowJo

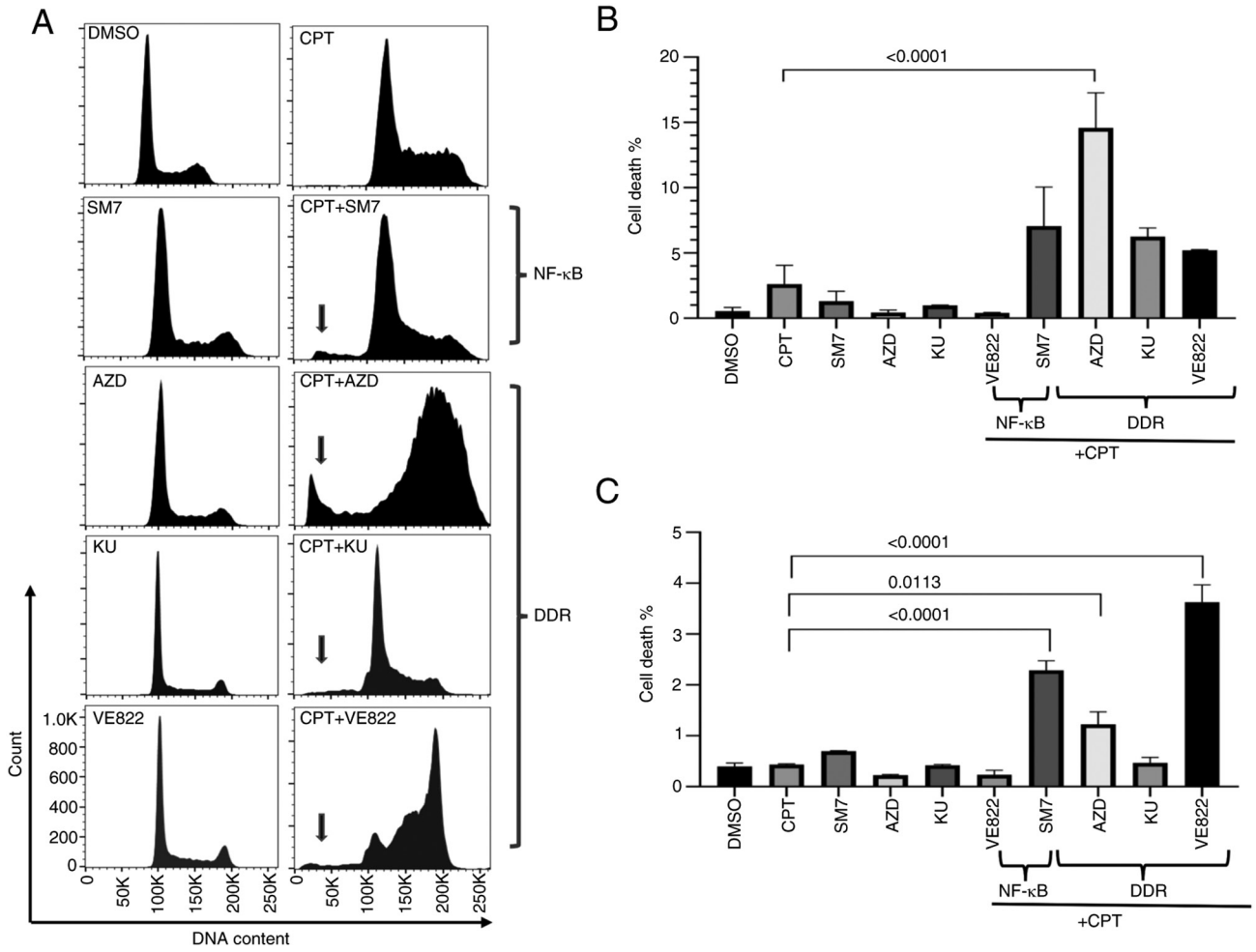


Figure 1. Inhibition of DDR pathways in CPT-treated cells enhances cell death. SW620 cells were treated with vehicle (DMSO), single agents (CPT, SM7, AZD, KU or VE822 at the concentrations indicated in materials and methods), or a combination of CPT and the pathway-inhibitor drugs as shown. Cell cycle profiles of treated cells were analyzed by propidium iodide flow cytometry. Shown are (A) representative histograms from SW620 cells and graphs from technical replicates of the experiment from (B) SW620 and (C) HCT116 cells. Arrows point to the to the sub-$G_1$ population indicative of cell death. AZD, AZD7762; DMSO, dimethyl sulfoxide; CPT, camptothecin; DDR, DNA-damage response; KU, KU60019; SM7, SM-7366.

Collectors' Edition (FJCE) version 7.5.109 (Cytek Biosciences, Fremont, CA, USA). Data analysis was done by using FlowJo versions 7.5 or 10.4 (BD Biosciences, Ashland, OR, USA).

*Statistical analysis*. Statistical comparisons of treatment effects were performed using one-way ANOVA followed by Dunnett's multiple comparisons test in GraphPad Prism software (version 9.4.0) for Windows (GraphPad Software, Inc.). Median fluorescence intensity values were used to create comparison graphs. Error bars, where plotted, show standard deviations of samples in triplicates. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Inhibition of DDR and NF-κB pathways in topo-inhibited cells accelerates cell death*. Camptothecin (CPT) and its derivatives interfere with topoisomerase, halting DNA replication and therefore cell division. However, DNA damage repair (DDR) competent cells could repair the damage and maintain cell viability. Prior studies have also shown that colon cancer cells exposed to topoisomerase inhibitors activate pathways that modulate cell survival and immune cell functions, including DDR response and NF-κB activation. To examine the effects of interference with these two pathways in colon cancer cells exposed to CPT, we determined cell cycle and cell death parameters for SW620 (MSS) or HCT116 (MSI) cells co-treated with CPT and selective inhibitors of the two pathways (SM-7368 as NF-κB pathway inhibitor; and AZD7762, KU60019 and VE822 as DDR pathway inhibitors targeting either Chk kinase, ATM kinase or ATR kinase, respectively). To best delineate the effects of the combinations, the cells were treated with individual drug concentrations that did not induce massive death. Fig. 1A shows a representative histogram from SW620 cells treated with control, single agents and combinations of the drugs. As shown in Fig. 1A-C, while CPT, SM-7368 (SM7) or AZD7762 (AZD), KU60019 (KU), or VE822 individually did not increase the sub-G1 population in both cell types at the concentrations we used, combination treatments markedly increased the sub-G1 population indicative of apoptotic cell death. Therefore, inhibition of both the DDR pathways activated by checkpoint kinases, and NF-κB activated in response to DNA-targeted therapeutics may augment cell death induced by drugs that interfere with

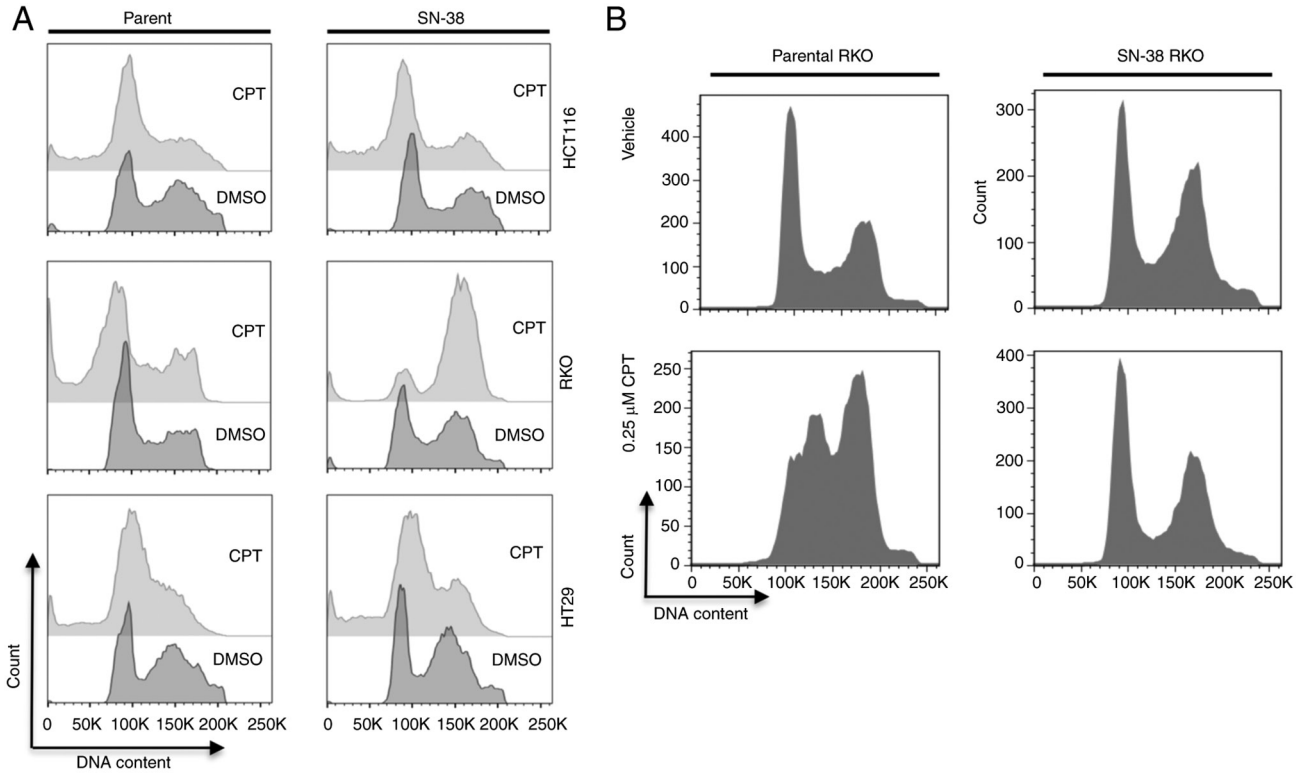


Figure 2. Acquired tolerance to SN-38 alters the cell cycle response to CPT treatment. Parental or SN-38 tolerant HCT116, RKO or HT-29 cells were treated with CPT. Cell cycle profiles of (A) all three cell lines treated at 1 $\mu$M concentration or (B) RKO cells treated at 0.25 $\mu$M concentration for 24 h are shown. While HCT116 and HT-29 cells exhibited a profile consisting of reduced $G_2$/M population with increase in $G_1$/S and sub-$G_1$ population, RKO cells responded with a $G_2$-shifted response, the difference between the parental and tolerant cells being the drug concentration threshold needed for similar response. CPT, camptothecin.

topoisomerase enzyme. HCT116 (MSI) cells treated similarly also showed increased sub-G1 although the relative amount of cell death due to the combination treatment was lower (Fig. S1), and NF-κB inhibition or ATR appeared to induce more cell death in these cells (Fig. 1C).

*Adaptive tolerance through extended exposure to topoisomerase inhibitor irinotecan (SN-38) may alter cell cycle response of colon cancer cells*. Adaptive resistance to cancer therapy agents is known to impact drug efficacy and recurrence. To simulate these characteristics, we generated SN-38 (irinotecan)-tolerant cells from HCT116 (MSI), RKO (MSI) or HT-29 (MSS) cell lines by continuous exposure of the cells to SN-38 over a period of 8 months as described in materials and methods. We used these cells to examine the cell cycle response after treatment for 24 h with 1 $\mu$M CPT, a concentration at which peak NF-κB signaling activity was detected in many treated colon cancer cells (29,30). As a result (Fig. 2A), we did not find marked differences in the cell cycle profiles and sub-G1 population profiles for parental or SN-38 tolerant HCT116 and HT-29 cells. However, while parental RKO cells displayed a high percentage of sub-G1 population, the SN-38-tolerant cells arrested in G2 with no increase in cell death (sub-G1 population). When we reduced the concentration of CPT to 0.25 $\mu$M for these cells, the parental RKO cells predominantly arrested in S-G2 phase of the cell cycle, whereas the SN-38 tolerant cells showed no cell cycle response, displaying profiles indistinguishable from the parental cells (Fig. 2B). Therefore, at least in RKO cells, the threshold for G2-shifted response was elevated by acquired tolerance to topoisomerase inhibition. This suggested that adaptive resistance or tolerance to topoisomerase inhibition may alter, though not universally, the drug response of cells upon exposure to a higher concentration of drugs of the same mechanism of action. As both HCT116 and RKO cells are MSI, yet they differ in their responses, it is not obvious if MS status is the factor for the observed difference between the two.

*Adaptive tolerance through extended exposure to topoisomerase inhibitor irinotecan (SN-38) may alter expression of PD-L1 in colon and colorectal cancer cells*. We and others have previously shown that colon cancer cells increase the surface expression of PD-L1 upon treatment with DNA-damaging drugs (30-34). Here, we undertook experiments to determine the impact of extended exposure to SN-38 on the expression of this immunoregulatory protein. First, we established the surface detection of PD-L1 by immunofluorescence staining of RKO cells that abundantly express PD-L1 (Fig. 3A). We then verified that topoisomerase I or II inhibitors, i.e., CPT, IRI (both inhibit topo I) or VP-16, (topo II) all induced the upregulation of PD-L1 in SW620 cells. In parallel, the cells were treated with 5-FU a nucleoside analog that interferes with DNA replication and RNA transcription. Gamma-interferon, a known inducer of immune regulatory genes, was also included (Fig. 3A and B). Additionally, immunoblotting of cell lysates prepared from

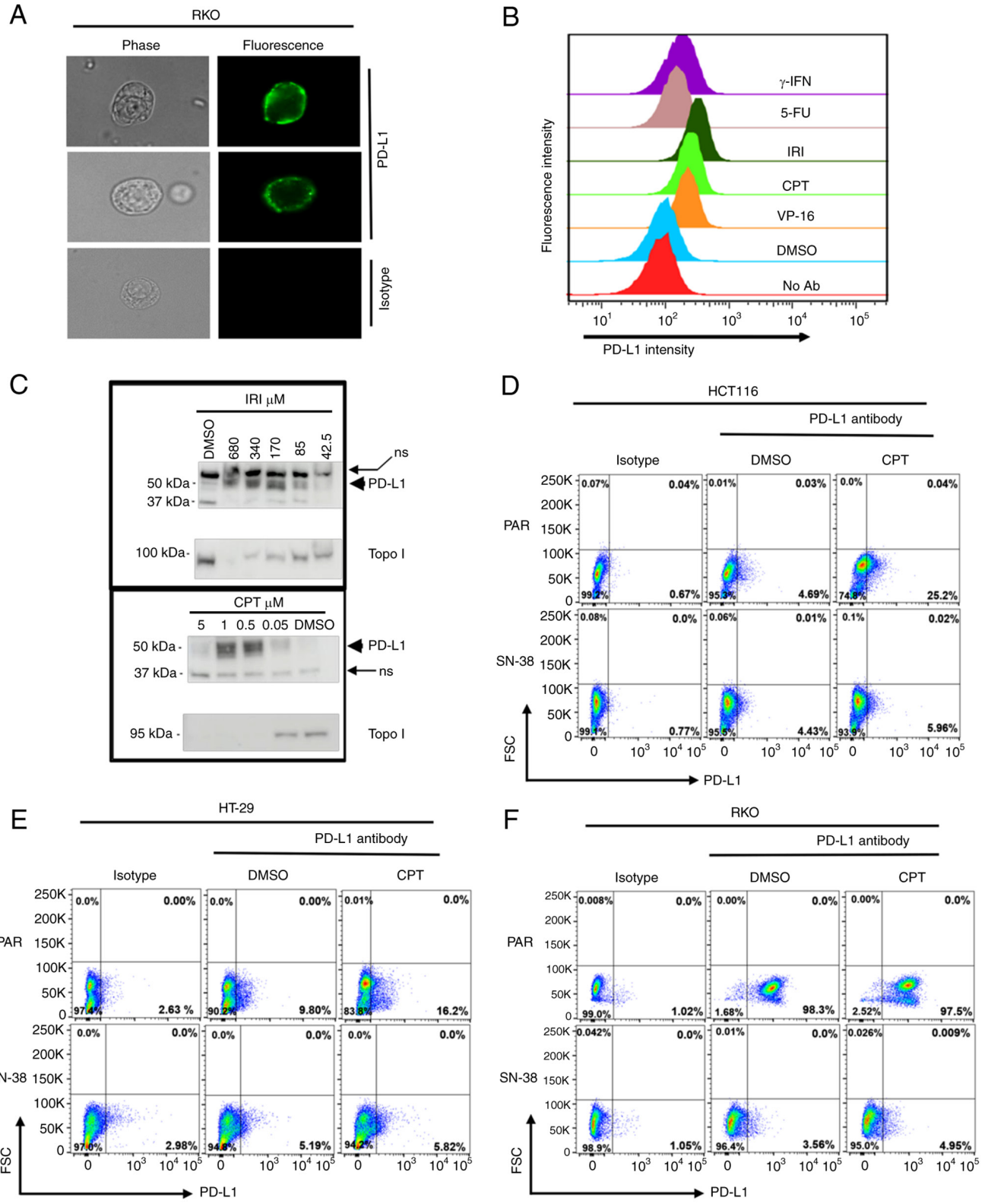


Figure 3. PD-L1 surface expression in response to treatment is reduced in cells that acquire tolerance to topoisomerase inhibition. (A) Detection of PD-L1 on the surface of colon cancer cells; two RKO cells stained with specific antibody (upper two rows) and one stained with isotype control (bottom row) are shown. Phase contrast (left columns) and fluorescence (right columns) microphotographs of the cells were taken at a magnification of x400. Topoisomerase inhibitors (VP-16, CPT and IRI) are potent inducers of PD-L1 expression on cell surface. (B) PD-L1 staining intensity histograms from SW620 cells treated with the indicated drugs are shown. Histograms of the median fluorescence intensities are presented. (C) Concentration-dependent increase in PD-L1 expression following treatment with both CPT and irinotecan. Immunoblots from SW620 cells treated with IRI or CPT at the indicated concentrations are shown. Membranes were re-probed with Topo I antibody to show drug activity. ns, non-specific bands to show equivalent loading. (D-F) Flow cytometry comparison of cell surface expression of PD-L1 in Par or SN-38 tolerant (SN38) (D) HCT116, (E) HT-29 or (F) RKO cells treated with vehicle (DMSO) or CPT (0.25 $\mu$M) and stained with isotype or PD-L1 antibody. Dot plots for PD-L1 (x-axis) and FSC (y-axis) from one of three technical duplicates are shown. Note that gates for each cell type were set to capture comparable percentages of cells in the control (isotype) plots, to allow measurements of relative PD-L1 expressions under baseline (DMSO) treatment or drug (CPT) treatment conditions. CPT, camptothecin; FSC, forward scatter; IRI, irinotecan; Par, parental; PD-L1, programmed death-ligand 1; Topo I, topoisomerase I; γ-IFN, gamma interferon; 5-FU, 5-fluorouracil; No Ab, no antibody; ns, non-specific.

SW620 cells treated with varying concentrations of IRI or CPT was performed (Fig. 3C).

These results showed that topoisomerase inhibitors (IRI, CPT and VP-16 are strong inducers of PD-L1 and the induction of PD-L1 is concentration-dependent, whereby low or high concentrations did not induce expression. After establishing the PD-L1 induction and surface expression, we tested if adaptive tolerance to SN-38 would alter the PD-L1 surface expression in colon or colorectal cancer cells. Parental and tolerant cells were compared. As shown in Fig. 3D-F all three tested parental cells (HCT116, HT-29, RKO), showed PD-L1 upregulation to variable degrees upon exposure to IRI, but the SN-38 adapted cells showed no increase at the concentration and time to which the parental cells responded. This suggested that the mechanism of PD-L1 upregulation is linked to the mechanism of the drug activity, and adaptation to the inhibitor may lead to non-responsive state. Although this lack of increase in PD-L1 levels may seem to render the cancer cells readily targetable by immune cells, other factors beside the expression of PD-L1 may determine the outcomes of the interaction of these adapted cancer cells with immune cells.

*Interference with NF-κB and DDR pathways modestly alters the upregulation of surface PD-L1 by topoisomerase inhibition*. Next we wanted to evaluate the effect of inhibitors of NF-κB or DDR pathways on the upregulation of PD-L1 expression on the surface of topoisomerase inhibited colon cancer cells derived from MSS or MSI origin. For this, we used inhibitors of NF-κB (SM-7366), Chk kinase (AZD), ATM kinase (KU60019), or ATR kinase (VE822) either alone or in combination with IRI, and determined the surface expression of PD-L1 on colon cancer cells. As shown on Fig. 4, co-treatment of the MSS SW620 (A-B) or MSI HCT116 (C-D) cells with the inhibitors only modestly altered the surface expression of PD-L1 beyond that induced by irinotecan. Nevertheless, a consistent decrease in the median fluorescence intensity for PD-L1 was noticed in both cell types when Chk kinase inhibitor was combined with topoisomerase inhibitor (Fig. 4B and D).

*Topoisomerase inhibitor treatment enhances the expression of MHC I on colon cancer cells*. Cancers that arise from hypermutated cells are considered readily visible to immune cells, and therefore better candidates for immunotherapy. Immunologically, the visibility of such tumors is mostly dependent on the MHC I-enabled cell surface presentation of neo-antigens from mutant proteins. Therefore, MHC I expression on the targeted cells is critical for the cytotoxic effects of T-cells. To assess the levels of MHC I on colon cancer cells treated with topoisomerase inhibitors, we determined expression of MHC I on SW620 (MSS) cell line by using an antibody that recognizes pan-MHC I molecules expressed on human cells. Flow cytometry was used for the detection of surface expression. An isotype antibody of the same class and source species was used as control. As shown on Fig. 5A and B, the MHC I antibody not only distinctly detected surface MHC I proteins, but the surface expression of MHC I (stained by HLA I antibodies) was increased after treatment. Inhibitors of Topoisomerase I (CPT, IRI, Topotecan), topoisomerase II (VP-16), and the control cytokine IFN-gamma increased the surface abundance of MHC I. To check if other chemotherapy agents in colon cancer therapy also upregulated the expression of MHC I on cell surfaces, we included 5-FU, oxaliplatin, CPT, erlotinib, and taxol in similar assays. Fig. 5C and D show the results of these experiments, where the isotype antibody did not detect MHC I in treated cells (Fig. 5C) and the variable effects of the other drugs on MHC I (Fig. 5D). The results also show that while CPT was the most potent inducer of MHC I, 5-FU, Oxaliplatin and Taxol induced MHC I by only 13-19%, while erlotinib, an EGFR inhibitor did not induce an increase under the same conditions. A similar increase was also evident in additional colon cancer cell lines (shown below in Fig. 6). These findings suggest that exposure to DNA-damage inducing drugs and taxol, which causes chromosomal mis-alignment and -segregation, may lead to upregulation of MHC I in colon cancer cells.

*Interference with NF-κB and DDR pathways modulates the upregulation of MHC I expression induced by topoisomerase inhibition*. Once we established the upregulation of MHC I on cell surface after topoisomerase inhibition, we wanted to examine if such upregulation is modified by inhibitors of NF-κB (SM-7366), Chk1 and Chk2 (AZD), ATM (KU60019), or ATR (VE822). Pathways inhibited by these drugs are mediators of DDR and are targets of clinical or investigational therapeutics. Inhibitors were tested individually or in combination with IRI, and MHC I expression was evaluated by flow cytometry.

As shown in Fig. 6A-C, NF-κB inhibitor (SM-7366) consistently enhanced the surface expression of MHC I when combined with IRI, while it did not have any enhancing effect by itself (not shown). On the other hand, combination of AZD (chk1 and chk2 inhibitor) with IRI consistently suppressed MHC I expression in the three cell lines we tested. ATM inhibitor (KU60019) caused moderate to marked reduction in the expression of MHC I in all three cell lines. On the other hand, ATR inhibitor (VE822) showed variable effect depending on the cell type, i.e. no change for SW620, moderate reduction in RKO, or marked decrease in HT-29. These results showed that depending on the pathway inhibited, the effect of topoisomerase inhibition on MHC I expression may be enhanced, reduced, or remain unchanged. Interestingly, in all three cell lines treated with the Chk-, or ATM-inhibitors or in HT-29 cells treated with the ATR-inhibitor VE822, we noticed not only lack of upregulation of MHC I but also an active downregulation of the surface MHC I. In these circumstances, the levels of MHC I on the surface was lower than the baseline levels or found in vehicle treated control cells, indicating a net reduction caused by the treatment. Taken together, these findings suggest that the levels of MHC I on cancer cells can be modulated by chemotherapeutic drugs and/or inhibitors, and this outcome may provide an opportunity to render the cells visible to immune cells in tumor microenvironment.

*Adaptive tolerance through extended exposure to topoisomerase inhibitor (SN-38) negatively impacts the expression of MHC I on colon or colorectal cancer cells*. Finally, to determine if acquired tolerance to topoisomerase inhibition alters the surface expression of MHC I, we compared parental

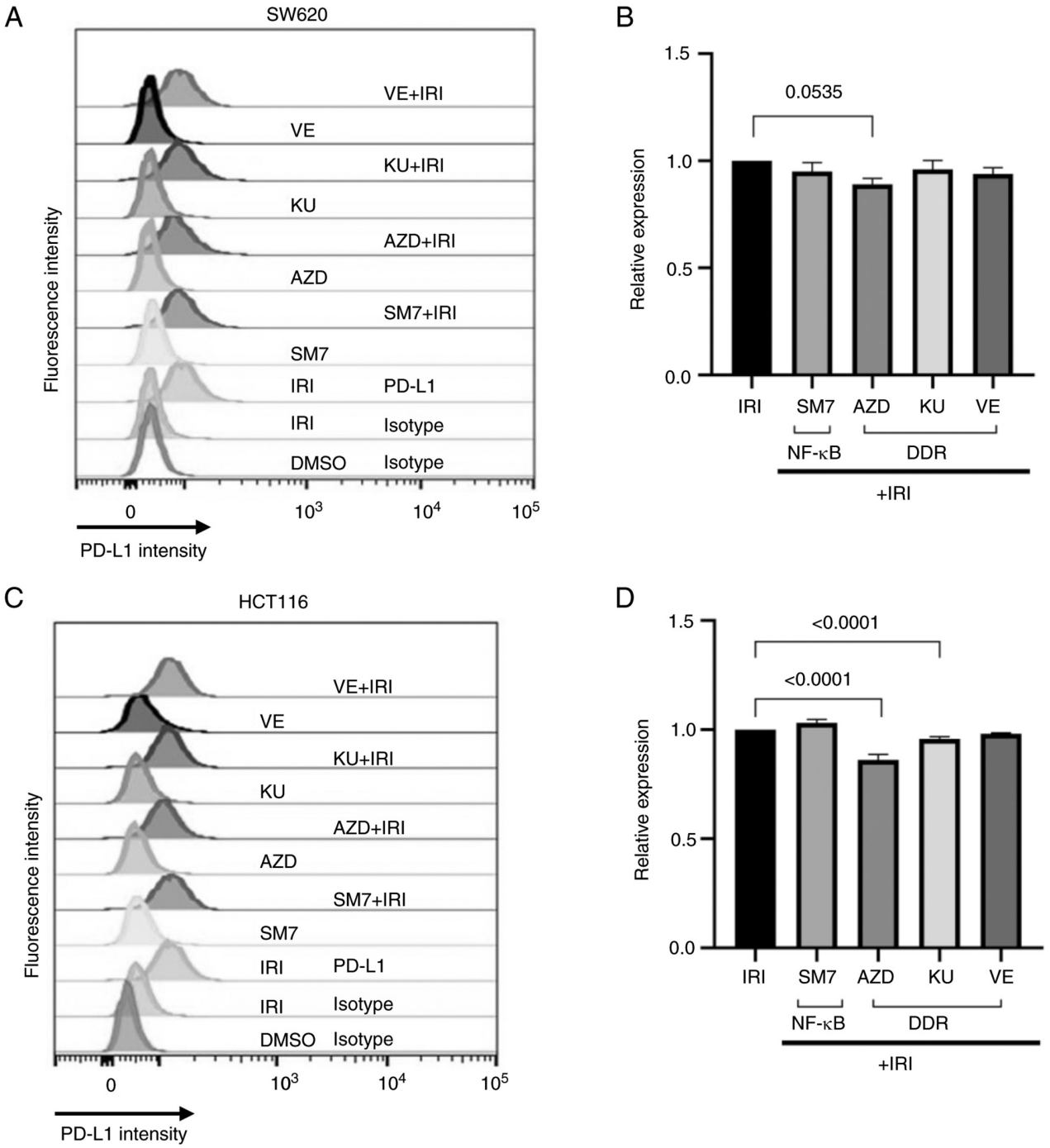


Figure 4. NF-κB or DDR inhibitors only moderately alter the induction of PD-L1 by IRI. (A and B) SW620 or (C and D) HCT116 cells were treated with vehicle (DMSO), IRI, NF-κB inhibitor (SM7), Chk kinase inhibitor (AZD), ATM inhibitor (KU) or ATR inhibitor (VE) as single agents or in combination with IRI (+IRI). (A and C) Surface expression of PD-L1 was measured by flow cytometry using PD-L1-specific antibodies. DMSO or IRI-treated cells were stained using a control (isotype) antibody to control for background or non-specific reactions. (B and D) PD-L1 expression in combination-treated SW620 and HCT116 cells, respectively, relative to the expression induced by IRI alone. Graphs were generated from three technical duplicates. Combination treatments did not increase PD-L1 expression beyond the levels obtained by IRI treatment, but the combination of IRI with AZD moderately reduced the relative expression in both cell types. DDR, DNA-damage response; IRI, irinotecan; SM7, SM-7368; Chk, checkpoint; AZD, AZD7762; KU, KU60019; VE, VE822; PD-L1, programmed death-ligand 1; ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia mutated and Rad3-related.

HCT116 (MSI), RKO (MSI) or HT-29 (MSS) against adaptively SN-38 tolerant cells derived from the three cell lines. We treated the cells with IRI concentrations that enhanced MHC I expression in parental cells and determined the surface expression of MHC I by flow cytometry. As shown in Fig. 7A-C, in all the three cell lines we tested, the percent increase in MHC I expression was much lower in adaptively tolerant cells compared to the parental cells under the same treatment conditions.

## Discussion

In this study we show that clinically used topoisomerase inhibitor drugs have the potential to alter the expression

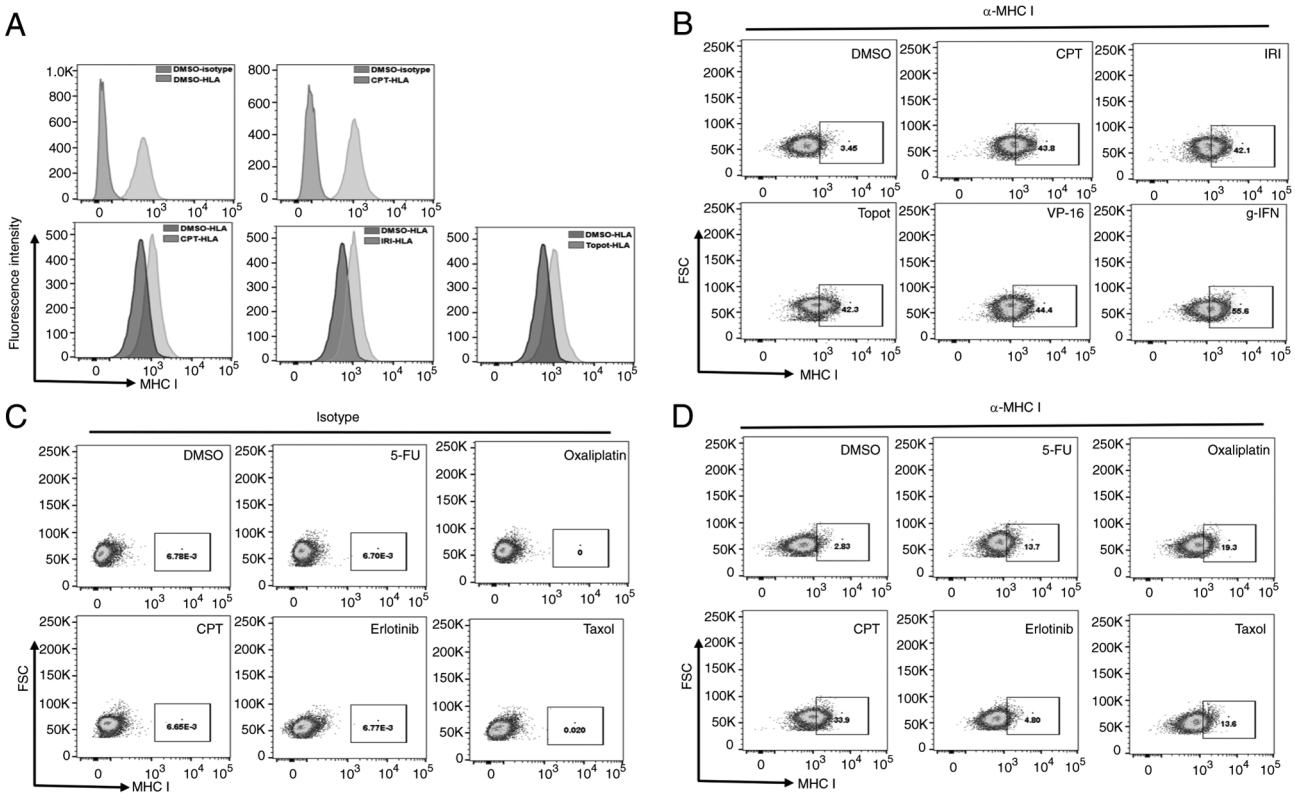


Figure 5. MHC I surface expression is increased in colon cancer cells treated with chemotherapy drugs. (A and B) Increased surface expression induced by topoisomerase I inhibitor drugs CPT, irinotecan and topotecan as shown by (A) histogram or (B) dot plots comparing isotype and HLA I-antibodies in vehicle-treated (DMSO) or drug-treated SW620 cells. (C and D) SW620 cells were treated with vehicle (DMSO) or the drugs 5-FU, oxaliplatin, CPT, erlotinib or taxol. Surface expression of MHC I was analyzed by flow cytometry using (C) isotype control or (D) pan-MHC I antibody. CPT, camptothecin; IRI, irinotecan; MHC, major histocompatibility complex; HLA, human leukocyte antigen; 5-FU, 5-fluorouracil; FSC, forward scatter; γ-IFN, γ interferon; Topot, topotecan.

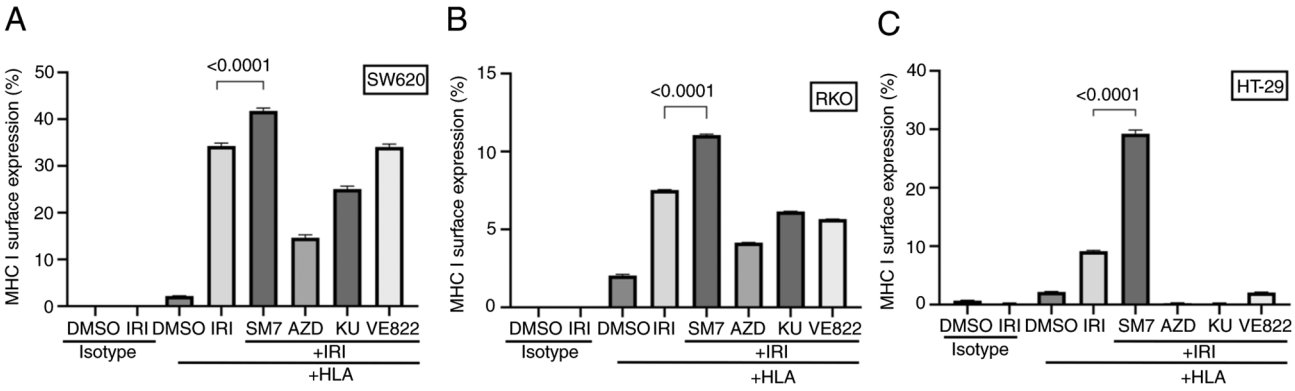


Figure 6. Interference with the NF-κB pathway enhances the surface expression of MHC I while interference with DDR pathways suppresses the expression. RKO, SW620 or HT-29 cells were treated with vehicle (DMSO) or IRI in combination with or without the addition of SM7, AZD, KU or VE822 pathway inhibitors. Shown are the MHC I surface staining median fluorescence graphs from technical triplicates for (A) RKO, (B) SW620 or (C) HT-29 cells. While NF-κB inhibition consistently enhanced the expression of MHC I in IRI-treated cells, DDR pathway inhibitors appeared to interfere with the surface expression of MHC I. DDR, DNA-damage response; IRI, irinotecan; MHC, major histocompatibility complex; SM7, SM-7368; AZD, AZD7762; KU, KU60019; HLA, human leukocyte antigen.

of cell surface molecules that may make colon cancer cells visible to immune cells. Firstly, we confirm that the protein PD-L1 is upregulated most potently by both topoisomerase I and II inhibitors and is detectable on cell surfaces. PD-L1 has been a molecule of most interest in immunotherapy because its blockage has enabled an unprecedented success in cancer therapy. Nevertheless, some types of cancer, including the majority of CRC, remain non-responsive for reasons yet to be defined. One of the approaches to enhance the chances of immunotherapy success is to convert 'cold' or invisible tumors to 'hot' or immune-visible tumors by manipulating cancer cells or their microenvironment. Studies are ongoing to understand mechanism for invisibility of tumors such as MSS colorectal cancer. Several combination therapies are also being tested to

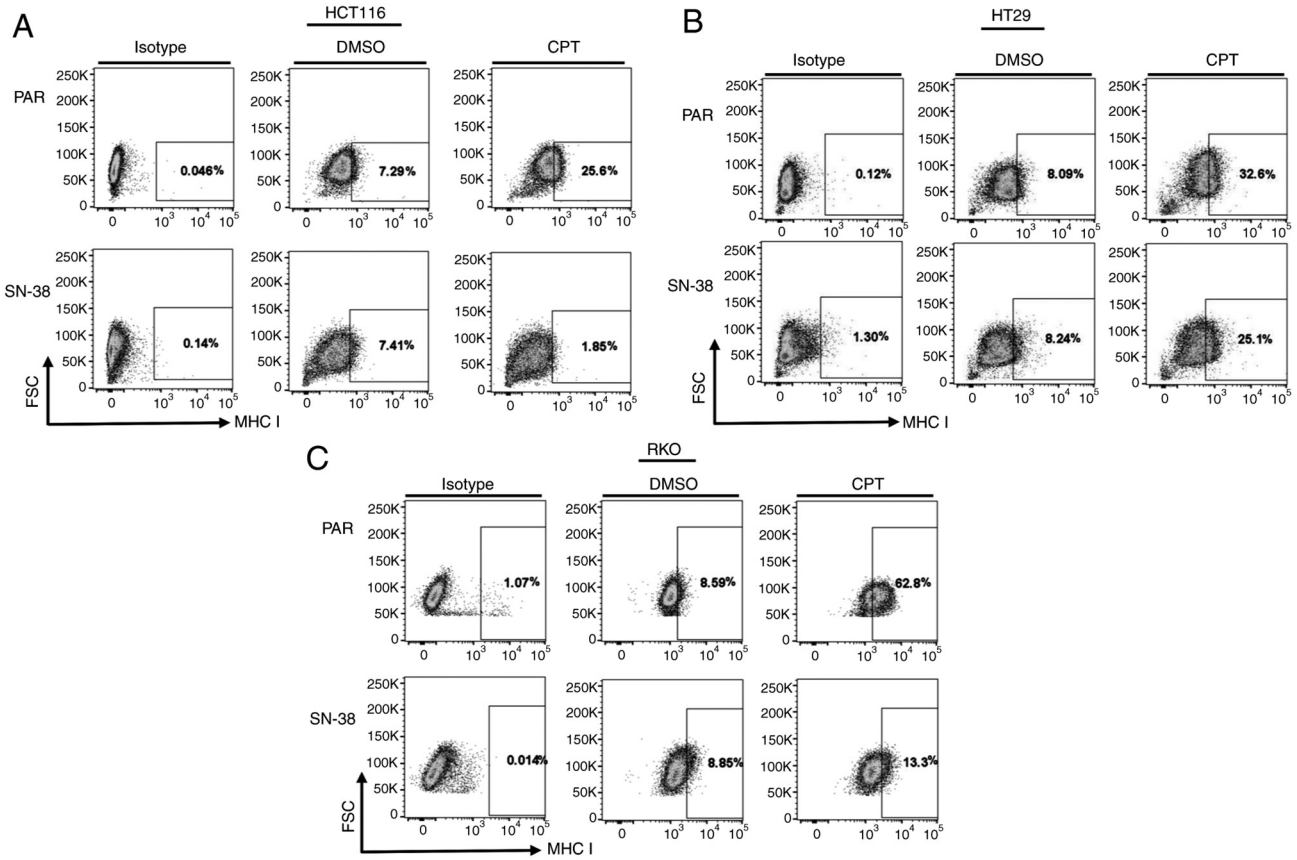


Figure 7. Acquired tolerance to topoisomerase inhibitor moderates the cell surface expression of MHC I molecules induced by treatment. PAR or tolerant (SN-38) (A) HCT116, (B) HT-29 or (C) RKO cells were treated with vehicle (DMSO) or 0.5 $\mu$M CPT for 24 h. Flow cytometry was used to detect the surface expression of MHC I using isotype- or pan MHC I-antibody. Gates for analysis of each cell type were set to allow a baseline of 7-9% of the vehicle-treated cells in the rectangles, and to compare the increase in expression beyond the relative baseline. Plots from one of three technical replicates for each cell line are shown here. Note that gates for each cell type are set to capture comparable baseline (DMSO) expression, to allow measurements of changes in MHC I expression due to drug (CPT) treatment. CPT, camptothecin; MHC, major histocompatibility complex; PAR, parental; FSC, forward scatter.

enhance the potency of chemotherapy drugs by exploiting the potential of DNA damaging drugs to generate neoantigens. However, the results of such therapies are not yet available, and the fundamental reason the cancer cells are invisible remains incompletely understood.

Prior studies using various CRC, mammary, prostate, and other cancer models (31-36) have shown the upregulation of PD-L1, as well as MHC I and peptide presentation by drugs that target critical signaling pathways, including topoisomerase functions. Although MSS tumors constitute the majority of CRC cases non-responsive to therapy, challenges remain to study immunotherapy outcomes in MSS tumors, primarily due to lack of suitable pre-clinical models. Here, we show that the expression of both PD-L1 and MHC I is enhanced by treatment with topoisomerase inhibitor drugs in both MSI and MSS cell types. Given the critical function of MHC I to present cellular protein breakdown peptides and neoantigens on the surface of cells, it is reasonable to hypothesize that the newly expressed MHC I molecules on cancer cells may contribute to the recognition by cytotoxic T-cells in the microenvironment. In this regard, we also show for the first time that drug-tolerant cells react differently compared to naïve cells. Taken together, these observations make clinical studies on the nature of immunomodulation by both MSS and relapsed tumors very critical and impactful.

An important question about enhanced MHC I expression is, what the peptides displayed on the newly expressed MHC I proteins might be. Since an MHC molecule can bind different varieties of peptides, it is not expected for a single peptide to be displayed on the newly expressed MHC molecules. However, the enhanced expression of these molecules correlates with an enhanced turnover or generation of peptides, and therefore, the chances will be greater for CD8+ T cells in the microenvironment to recognize the neoantigens. However, the simultaneously increased expression of PD-L1 on treated cancer cells may initiate a negative reaction on T-cells, muting their activity on tumor cells. Nevertheless, knowledge about the effect of a topoisomerase-targeted treatment on the abundance of MHC I molecules on tumor cells may be helpful to selectively identify individuals who may best benefit from chemotherapy-immunotherapy combination.

Combination of chemotherapy with signaling pathway inhibitors has been considered an alternative to enhance the efficacy of cytotoxic drugs, while helping to reduce the dose needed to achieve the same outcome. Therefore, we tested if the combination of chemotherapy with specific pathway (NF-κB or DDR) inhibitors would augment the effects of irinotecan, a clinically used drug to treat colorectal cancer. Irinotecan enhances the surface abundance of both PD-L1- and MHC I on colon cancer cells. Our results from *in vitro* studies show

that the simultaneous inhibition of topoisomerase and NF-κB pathway consistently upregulated the surface expression of MHC I, while inhibition of neither NF-κB nor DDR pathways affected the PD-L1 expression induced by topoisomerase inhibition. On the other hand, inhibition of ATM or Chk 1/2 consistently interfered with the increase in MHC I induced by topoisomerase inhibitor treatment. Although inhibition of either of NF-κB or DDR inhibitors in topoisomerase inhibited cells may induce apoptotic cell death, immune regulatory outcomes of the combination-treatment may be different between NF-κB and DDR inhibitors. The preliminary results shown here suggest that *in vivo* studies need to be performed to identify if inhibition of NF-κB pathway is more effective than inhibition of DDR pathways to turn immunologically cold colon cancer microenvironment into a 'hot' one.

This suggests that immunotherapy that relies on MHC I presentation of neoantigens may be challenged if cancer cells acquire resistance to the chemotherapy. On the other hand, since loss of MHC I renders cells visible to NK-cells, which also play role in immunotherapy, alternative approaches will be needed to treat such resistant cancers by activating resident NK-cells.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

TS, PD and CY conceived and designed the study. MH, VT, DB, SS, DG and TS were involved in experiments, and data collection and analysis. MH, TS and PD wrote and revised the manuscript. TS and MH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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