OXFORD

JNCI J Natl Cancer Inst (2018) 110(7): djx257

doi: 10.1093/jnci/djx257
First published online December 18, 2017
Article

ARTICLE

The Effect of Topoisomerase I Inhibitors on the Efficacy of T-Cell-Based Cancer Immunotherapy

Jodi A. McKenzie, Rina M. Mbofung*, Shruti Malu*, Min Zhang, Emily Ashkin, Seram Devi, Leila Williams, Trang Tieu, Weiyi Peng, Sunila Pradeep, Chunyu Xu, Soraya Zorro Manrique, Chengwen Liu, Lu Huang, Yuan Chen, Marie-Andree Forget, Cara Haymaker, Chantale Bernatchez, Nikunj Satani, Florian Muller, Patrick Hwu

Affiliations of authors: Department of Melanoma Medical Oncology (JAM, RMM, SM, EA, LW, WP, CX, SZM, CL, LH, YC, MAF, CH, CB, PH), Department of Lymphoma/Myeloma (MZ), Department of Sarcoma Medical Oncology (SD), Institute for Applied Cancer Science (TT), Department of Gynecologic Oncology and Reproductive Medicine (SP), and Department of Cancer Imaging Systems (NS, FM), The University of Texas MD Anderson Cancer Center, Houston, TX.

*Authors contributed equally to this work.

Please see the Notes section for the full list of authors and affiliations.

Correspondence to: Patrick Hwu, MD, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 421, Houston, TX 77030 (e-mail: phwu@mdanderson.org).

Abstract

Background: Immunotherapy has increasingly become a staple in cancer treatment. However, substantial limitations in the durability of response highlight the need for more rational therapeutic combinations. The aim of this study is to investigate how to make tumor cells more sensitive to T-cell-based cancer immunotherapy.

Methods: Two pairs of melanoma patient-derived tumor cell lines and their autologous tumor-infiltrating lymphocytes were utilized in a high-throughput screen of 850 compounds to identify bioactive agents that could be used in combinatorial strategies to improve T-cell-mediated killing of tumor cells. RNAi, overexpression, and gene expression analyses were utilized to identify the mechanism underlying the effect of Topoisomerase I (Top1) inhibitors on T-cell-mediated killing. Using a syngeneic mouse model ($n = 5$ per group), the antitumor efficacy of the combination of a clinically relevant Top1 inhibitor, liposomal irinotecan (MM-398), with immune checkpoint inhibitors was also assessed. All statistical tests were two-sided.

Results: We found that Top1 inhibitors increased the sensitivity of patient-derived melanoma cell lines ($n = 7$) to T-cell-mediated cytotoxicity ($P < .001$, Dunnett's test). This enhancement is mediated by TP53INP1, whose overexpression increased the susceptibility of melanoma cell lines to T-cell cytotoxicity (2549 cell line: $P = .009$, unpaired t test), whereas its knockdown impeded T-cell killing of Top1 inhibitor–treated melanoma cells (2549 cell line: $P < .001$, unpaired t test). In vivo, greater tumor control was achieved with MM-398 in combination with α-PD-L1 or α-PD1 ($P < .001$, Tukey's test). Prolonged survival was also observed in tumor-bearing mice treated with MM-398 in combination with α-PD-L1 ($P = .002$, log-rank test) or α-PD1 ($P = .008$, log-rank test).

Conclusions: We demonstrated that Top1 inhibitors can improve the antitumor efficacy of cancer immunotherapy, thus providing the basis for developing novel strategies using Top1 inhibitors to augment the efficacy of immunotherapy.

Received: May 22, 2017; **Revised:** September 30, 2017; **Accepted:** November 8, 2017

Cancer immunotherapy, which aims to harness the power of the immune system to target and eradicate cancer cells, has been an area of keen research in oncology for several decades. However, the emergence of clinical data in the past several years demonstrating the potency of immunotherapy to increase the overall survival of cancer patients (1–5) has heightened the prominence of immunotherapy and led to the approval of a number of checkpoint inhibitors in several cancer indications. Objective response rates of up to 45% have been achieved with PD1/-L1-targeting antibodies in patients with metastatic melanoma, renal cell carcinoma, and non–small cell lung cancer (6–8). Despite these achievements, the full potential of cancer immunotherapy has not been realized, as most immunotherapy-treated cancer patients show little to no clinical benefit (9). The potency of cancer immunotherapy is undermined by immunoresistance mechanisms, either inherent or acquired as tumors seek to evade the immune response.

Recent studies from our group and others have elucidated some of the underlying mechanisms of immunoresistance. We have shown that PTEN loss inhibits T-cell-mediated killing and tumor T-cell infiltration and is correlated with poor outcomes in anti-PD-1-treated melanoma patients (10). Others have shown that activation of Wnt/β-catenin is associated with a non-T-cell-inflamed state in melanoma and is correlated with resistance to immune checkpoint blockade (11). Additionally, analysis of tumors from melanoma patients who progressed on anti-PD-1 therapy revealed that acquired resistance to PD-1 blockade was correlated with defects in interferon receptor signaling and in antigen presentation (12).

The current limitations of cancer immunotherapy highlight the need to better understand the molecular factors driving tumor response or resistance to immunotherapy. New and rational treatment strategies need to be developed to improve on current outcomes with single-agent immune checkpoint blockade. One such strategy is combination therapy involving different types of cancer immunotherapy (eg, antibodies, adoptive T-cell therapy) or combinations of immunotherapy with standard treatment options (eg, surgery, radiation, and chemotherapy).

In an effort to develop novel combination strategies for improving response to T-cell-based cancer immunotherapy, we completed a compound screen to identify bioactive agents that can increase T-cell-mediated cytotoxicity of tumor cells. We utilized our unique set of melanoma patient-derived tumor cell lines and their autologous TILs as a model system to assess T-cell-mediated killing of tumor cells, which is the ultimate effector function of cytotoxic T cells. We set out to determine if identified bioactive hits could have a synergistic effect on T-cell-mediated cytotoxicity of tumor cells, and if the combination with T-cell-based cancer immunotherapy would yield greater tumor control in vivo. The ultimate goal is to provide preclinical evidence to support the development of therapeutic strategies of immunotherapy-based combinations to improve clinical outcomes for cancer patients.

ARTICLE

Methods

Mice and Cell Lines

C57BL/6 female mice (6–12 weeks old) were obtained from the Charles River Frederick Research Model Facility (Bethesda, MD). Mice were housed under specific pathogen-free conditions, and experiments were performed in accordance with the requirements of the Institutional Animal Care and Use Committee. All patient-derived melanoma cell lines and their autologous tumor-infiltrating lymphocytes (TILs; n = 8 pairs) were generated under an institutional review board–approved laboratory protocol with required patient informed consent (LAB06-0755) as previously described (13). The MC38/gp100 cell line was generated as previously described (14). All cell lines were routinely tested for mycoplasma contamination and verified by short tandem repeat DNA typing. Cell culture details are provided in the Supplementary Materials (available online).

Drug Screen and Cytotoxicity Assay

Patient-derived melanoma cell line 2549 was screened using an 850 compound library. Tumor cells were first stained with DDAO (ThermoFisher; Waltham, MA); 5×10^4 labeled cells were incubated with 1 μM of each compound for 24 hours. Drug-treated cells were then washed and incubated for three hours with autologous TILs at predetermined effector to target cell ratios. Cells were then stained for flow cytometry analysis of activated caspase 3 as a readout for apoptosis. Cells treated only with drug, or autologous TILs, were also assessed. A comboscore was then used to evaluate the effect of the combination of each drug and TILs. The comboscore was calculated using the following formula: $[(\text{Apoptosis}_{drug+TILs} - \text{Apoptosis}_{drug})/(\text{Apoptosis}_{TILs})]^2$. For dose response studies, patient-derived cell lines were treated with SN38 or Topotecan prior to incubation with autologous TILs. CalcuSyn (BIOSOFT; Cambridge, UK) was then used to compute synergy.

Mouse Studies

Mice were inoculated with 5×10^5 MC38/gp100 cells and randomized into one of four treatment groups three days later: (i) control, (ii) MM-398, (iii) anti-PD1/-L1 antibody, (iv) MM-398 + anti-PD1/-L1. Treatment began on day 3, and all treatments were administered as follows: MM-398: 40 mg/kg i.v. once weekly; anti-PD-L1: 150 μg; or anti-PD1: 200 μg i.p. every three days. PBS was used as the diluent for both MM-398 and anti-PD1/L1. Rat IgG2B and Rat IgG2A antibodies were used as isotype-matched controls for anti-PD-L1 and anti-PD1, respectively. For analysis of immune cells, mice were inoculated with 5×10^5 MC38/gp100 cells and assigned to the four treatment groups. Treatment began seven days after tumor inoculation. Tumors were harvested 18 days after tumor implantation and used to generate single-cell suspensions for flow cytometry analysis, as previously described (14). Flow cytometry data were analyzed using FlowJo (FlowJo, LLC; Ashland, OR).

Statistical Analysis

Dunnett's multiple comparisons test was used to calculate the statistical significance of the effect of Topoisomerase I inhibitors on T-cell-mediated killing of patient-derived melanoma cell lines. The Fisher exact test was used to assess the statistical significance of the gene expression changes in Top1 inhibitor–treated melanoma cells. An unpaired two-tailed *t* test was used to compare continuous variables between two groups. Melanoma patient data were extracted from the publicly available The Cancer Genome Atlas database (16). The Kaplan-Meier method and log-rank test were used to compare the post-accession survival with respect to TP53INP1 expression (low vs high). The Mann-Whitney test was used to compare lymphocyte

score and cytolytic activity score between TP53INP1 low- and high-expression groups. Analysis of variance using Tukey's adjustment for multiple comparisons was used to compare tumor size and immune cell subsets, while the Kaplan-Meier method and log-rank test were used to compare survival between four treatment groups in in vivo experiments.

The combination index was calculated using CalcuSyn, which is based on the Chou-Talalay method of quantifying synergy, where combination index < 1 (synergism), > 1 (antagonism), or $= 1$ (additive) (15). Twenty-five compounds with the highest comboscores (>1.5), indicative of increased T-cell-mediated killing, were selected for further statistical analysis. A two-sample *t* test was then used to compare the apoptotic effect of the combination of each compound and TIL with the apoptotic effect of TIL alone. Ingenuity pathway analysis (QIAGEN; Valencia, CA) was used to generate a pathway heatmap showing the most statistically significantly altered signaling pathways in Top1 inhibitor–treated melanoma cells. Tableau (Tableau Software; Seattle, WA) was used to generate a gene expression heatmap of Top1 inhibitor–treated cells.

Statistical analyses were performed using GraphPad Prism v6.07 (GraphPad Software Inc.; La Jolla, CA). All statistical tests were two-sided. The threshold for statistical significance was a *P* value of less than .05, except for the drug screen, where a Bonferroni-corrected threshold of a *P* value of less than .002 was used to adjust for multiple testing. Exact *P* values are provided (unless $P < .001$).

Additional methods are provided in the Supplementary Materials (available online).

Results

T-Cell-Mediated Killing of Melanoma Tumor Cells

To identify bioactive agents that can increase T-cell-mediated killing of melanoma cells, the patient-derived melanoma cell line 2549 was utilized in a high-throughput screen of 850 compounds. To compute the effect of each compound on T-cell-mediated killing of melanoma tumor cells, a "comboscore" was calculated (Figure 1A). A comboscore greater than 1 was indicative of an increase in T-cell-mediated killing. Three inhibitors of Topoisomerase I (Top1), Topotecan Hydrochloride, Camptothecin, and Irinotecan Hydrochloride Trihydrate, scored as top hits from the screen, indicating that Top1 inhibitors could enhance T-cell-mediated killing of melanoma (Figure 1B). To validate results of the screen, several patient-derived melanoma cell lines were treated with SN38 (the active metabolite of irinotecan) or Topotecan prior to incubation with their autologous TILs. The results indicate that Top1 inhibitor treatment synergistically increased T-cell-mediated killing of melanoma cells (2338 cell line: SN38 alone [1 μM] mean = 15.53, SD = 1.894; TIL alone mean = 9.57, SD = 1.185; SN38+TIL mean = 31.92, SD = 1.552, combination index = 0.4; $P < .001$, Dunnett's test) (Figure 1, C–E; Supplementary Figures 1–2, available online). Specific lysis, as measured by chromium release, further validated these results, as a greater percentage of specific lysis was observed in melanoma tumor cells treated with both SN38 and autologous TILs, in comparison with cells treated only with TILs (Supplementary Figure 3, available online).

Gene Expression Analysis of Top1 Inhibitor-Treated Melanoma Cells

Having determined that Top1 inhibitors could increase T-cell-mediated killing of melanoma cells, we then sought to understand the molecular mechanisms underlying this phenotype. Four patient-derived melanoma cell lines were treated with SN38 or DMSO as a control and subsequently subjected to gene expression profiling by microarray analysis. Ingenuity pathway analysis (IPA) was used to assess the gene expression changes in SN38-treated cells in comparison with control cells. Comparative analysis in IPA showed that the TP53 pathway was the most statistically significant signaling pathway altered ($P < 0.001$, Fisher exact test) (Figure 2A) and that there was enrichment for increased expression of TP53-regulated genes in Top1 inhibitor–treated melanoma cells (Figure 2B). In particular, we observed consistent upregulation of the TP53 regulated factor tumor protein 53–inducible nuclear protein 1 (TP53INP1) in Top1 inhibitor–treated melanoma cells ($P = .02$ [2338, 2400, 2549]; $P = .03$ [2559], unpaired *t* test) (Figure 2C).

We then queried The Cancer Genome Atlas melanoma database for clinical factors associated with the expression of TP53INP1 in melanoma patients and found that stage III melanoma patients with high TP53INP1 expression had greater postaccession survival (n = 84 patients; mean = 97.6 months, 95% CI = 65.3 to N/A) in comparison with patients with low TP53INP1 expression (n = 84 patients; mean = 64.2 months, 95% CI = 34.8 to 115, $P = .03$, log-rank test) (Figure 3A). Postaccession survival is defined as the survival calculated from date of biospecimen collection/accession to date of last follow-up or death (16). We also found that in melanoma patients, higher TP53INP1 expression was associated with a higher lymphocyte score ($P < 0.001$, Mann-Whitney test), which is a semiquantitative measure of the number of lymphocytes in a sample (Figure 3B) (16). Additionally, the immune cytolytic activity score (17) was also statistically significantly higher in melanoma patients exhibiting higher levels of TP53INP1 ($P < .001$, Mann-Whitney test) (Figure 3C). Taken together, these data are indicative of a potential link between TP53INP1 and T-cell-mediated antitumor immune responses.

Functional Role of TP53INP1 in T-Cell-Mediated Cytotoxicity of Top1 Inhibitor-Treated Melanoma Cells

To determine the functional relevance of TP53INP1 to the enhancement of T-cell-mediated killing caused by Top1 inhibitors, we determined the effect of altered TP53INP1 expression on T cell cytotoxicity of melanoma cells. Increased T-cell-mediated killing was observed in TP53INP1-overexpressing cells (2549-TP53INP1+TIL: mean [SD] = 47.57 [0.3215]) in comparison with control cells (2549-GFP+TIL: mean [SD] = 28.93 [6.957]; $P = .009$, unpaired *t* test) (Figure 4, A and B). In contrast, knockdown of TP53INP1 (Figure 4C) impeded the effect of Top1 inhibitors on T-cell-mediated cytotoxicity of melanoma cells (2549-TP53INP1-shRNA: mean [SD] = 33.37 [1.043]; 2549-control-shRNA: mean [SD] = 50.84 [2.757]; $P < .001$, unpaired *t* test) (Figure 4D). These results indicate that TP53INP1 is necessary for Top1 inhibitor enhancement of T-cell-mediated killing of melanoma cells.

TP53INP1 is a TP53-responsive gene, being upregulated in response to genotoxic stress, and has been shown to be a positive regulator of TP53-induced apoptosis (18). We therefore asked if the enhancement in T-cell-mediated killing of melanoma tumor cells treated with a Top1 inhibitor is dependent on induction of

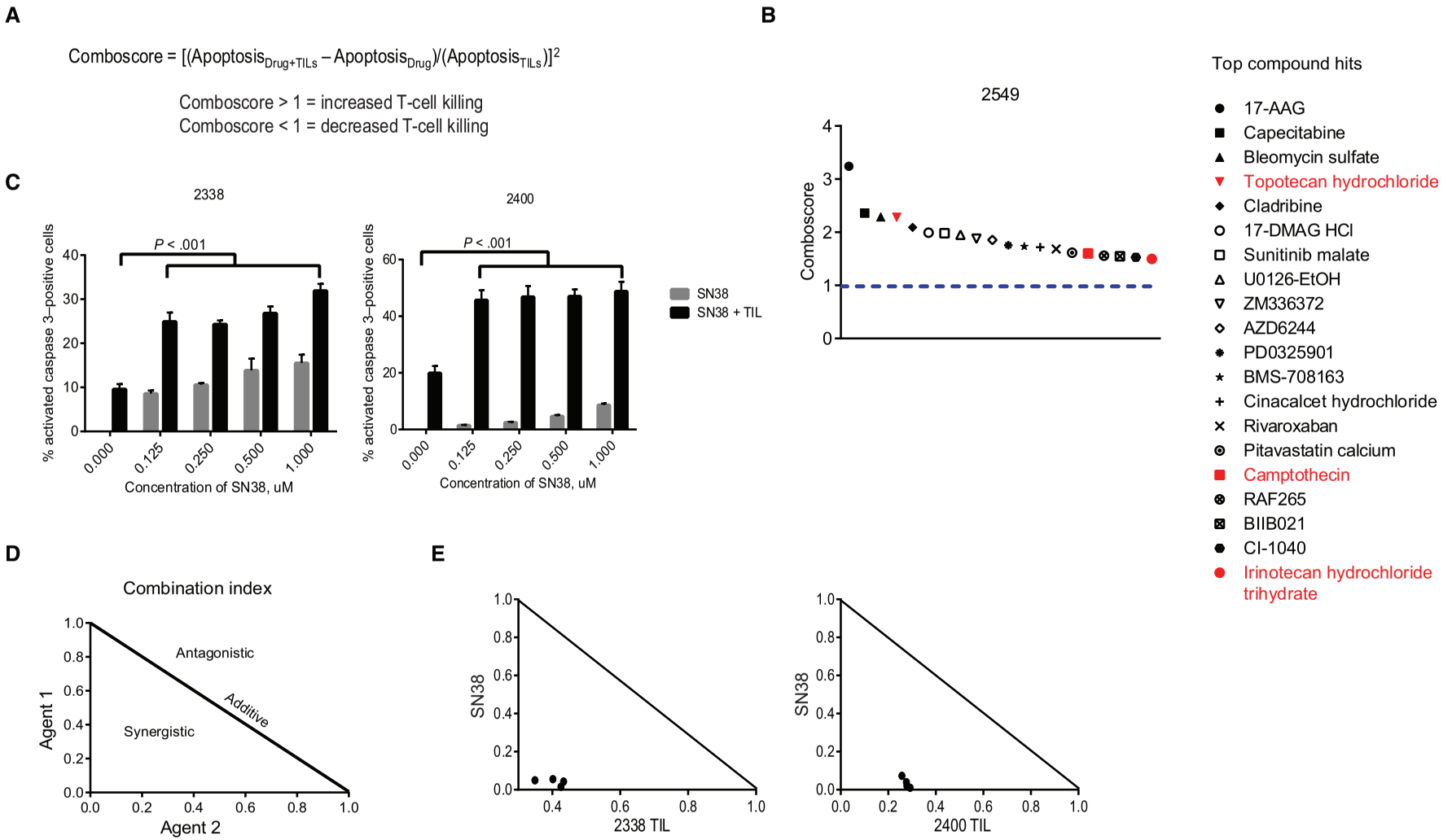


Figure 1. T-cell-mediated killing of Top1 inhibitor–treated melanoma cells. **A)** The comboscore developed to assess the effect of each bioactive agent on T-cell-mediated killing of tumor cells. **B)** Melanoma cell line 2549, along with its autologous tumor-infiltrating lymphocytes (TILs), was used in a high-throughput screen of 850 compounds. The top 20 hits from the screen are listed. Three Top1 inhibitors: Topotecan Hydrochloride, Camptothecin, and Irinotecan Hydrochloride Trihydrate (highlighted in **red**) were identified as hits from this screen based on having high comboscores and were ranked 4 out of 850, 16 out of 850, and 20 out of 850, respectively, among the 850 compounds assessed. **C)** The patient-derived melanoma cell lines 2338 and 2400 were treated with autologous TILs for three hours. Cells were then stained for activated caspase 3 to quantify apoptosis by flow cytometry; 2338 and 2400 cells were treated with the Top1 inhibitor SN38 for 24 hours at a concentration range of 0 to 1 μM. Cells were then stained for activated caspase 3, or drug-treated cells were washed and then incubated with autologous TILs for three hours. Apoptosis was then quantified as described. Data are mean ± SD. **D)** The software CalcuSyn was used to compute the combination index of combining SN38 with TIL. CalcuSyn is based on the Chou-Talalay method of quantifying synergy and is designed to compare the effect of two agents alone and in combination with each other to determine if their interaction is synergistic (combination index < 1), additive (combination index = 1), or antagonistic (combination index > 1) (15). **E)** The combination indexes of SN38 with 2338 TIL and 2400 TIL are represented in the normalized isobolograms. Data are representative of at least three independent experiments. All statistical tests were two-sided. *P* values were calculated using Dunnett's test. TIL = tumor-infiltrating lymphocytes.

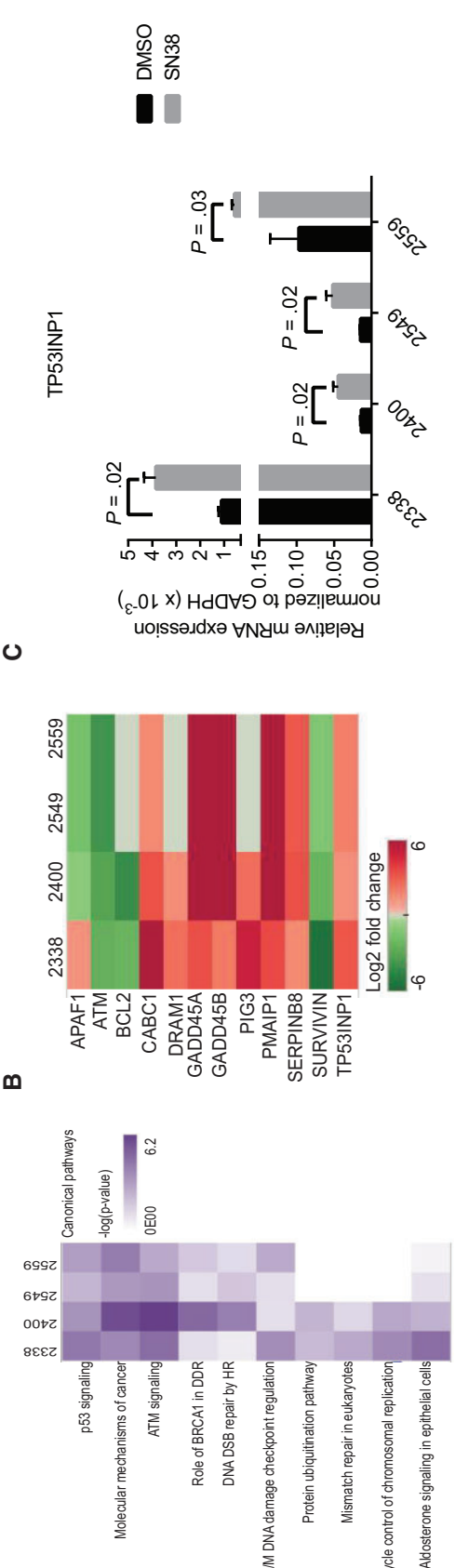


Figure 2. Gene expression changes in Top1 inhibitor–treated melanoma cells. **A)** Four melanoma cell lines were treated with 1 μM SN38 (or DMSO as control) for 24 hours and then subjected to gene expression analysis by microarray. Ingenuity pathway analysis was used to perform a comparative analysis of the gene expression changes. The top 10 most statistically significantly altered pathways are represented. **B)** Heatmap of differentially expressed TP53-regulated genes in SN38-treated melanoma cell lines. **C)** Quantitative real-time polymerase chain reaction validation of the upregulation of TP53INP1 in SN38-treated melanoma cell lines in comparison with controls (DMSO-treated cell lines). Data are mean ± SD. All statistical tests were two-sided. *P* values were calculated using the Fisher exact test (2A) and unpaired t test (2C). qRT-PCR = quantitative real-time polymerase chain reaction; TP53 = tumor protein 53; TP53INP1 = tumor protein 53–inducible nuclear protein 1.

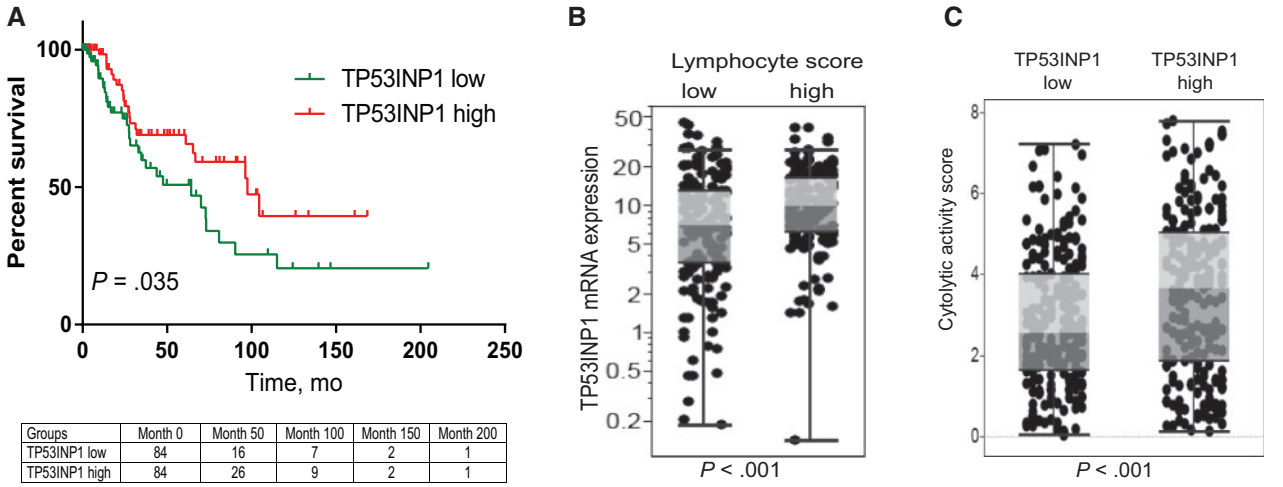
TP53 activity. siRNA was used to reduce the expression of TP53 in a melanoma cell line with wild-type TP53 (Supplementary Figure 4, A and B, available online). Knockdown of TP53 resulted in a statistically significant reduction in T-cell-mediated killing of SN38-treated melanoma cells (mean [SD] = 13.45 [3.391]) in comparison with T-cell-mediated killing of SN38-treated melanoma cells with intact expression of TP53 (mean [SD] = 37.62 [2.227]; $P < .001$, unpaired t test) (Figure 4E). Enhancement in T-cell-mediated killing was observed in seven of the eight human cell lines tested in our studies. In the eighth cell line, which carries an inactivating mutation in TP53, Top1 inhibition did not enhance T-cell-mediated killing (Supplementary Figure 4, A and C, available online). Taken together, these results suggest that the observed effect of Top1 inhibitor on T-cell-mediated cytotoxicity of melanoma tumor cells is dependent on TP53.

Antitumor Activity of the Combination of Liposomal Irinotecan and Immune Checkpoint Blockade

Having demonstrated that Top1 inhibitors can enhance T-cell-mediated cytotoxicity of tumor cells in vitro, we hypothesized that Top1 inhibitors could enhance the efficacy of immune checkpoint blockade. To test this hypothesis, we used the immunogenic MC38/gp100 mouse model. Tumor-bearing mice were treated with the Top1 inhibitor liposomal irinotecan (MM-398), an anti-PD1/-L1 antibody, or a combination of MM-398 and anti-PD1/-L1. Tumor-bearing mice treated with vehicle and isotype-matched control antibody served as controls (n = 5 per group). MM-398 was chosen for in vivo studies because of its pharmacokinetic and safety profile. Its encapsulated formulation allows for increased tumor retention by the enhanced permeability and retention effect, and decreased host toxicities (19). Greater tumor control was achieved with a combination of MM-398 and anti-PD-L1 (mean [SD] tumor volume = 40.04 [12.66] mm^3), in comparison with either MM-398 (mean [SD] tumor volume = 136.3 [64.77] mm^3; $P = .01$, Tukey's test) or anti-PD-L1 alone (mean [SD] = 373 [53.58] mm^3; $P < .001$, Tukey's test) (Figure 5A). Tumor-bearing mice treated with this combination also had the greatest overall survival (mean [SD] = 43.2 [8.643] days), in comparison with MM-398-treated (mean [SD] = 31.8 [5.02] days; $P = .02$, log-rank test) or anti-PD-L1-treated cohorts (mean [SD] = 26.4 [2.51] days; $P = .002$, log-rank test) (Figure 5B). Increased antitumor activity was also achieved with the combination of MM-398 and anti-PD1 (Figure 5, C and D).

To determine the effect of the combination treatment of MM-398 and anti-PD-L1 on immune cell subsets in vivo, immune cells were isolated from tumors and analyzed by flow cytometry to assess phenotype and function. The results demonstrated that the combination treatment increased tumor-infiltrating $CD8^+$ T cells in comparison with control or MM-398-treated cohorts, although there was no statistically significant difference when compared with the anti-PD-L1-treated group (Figure 6A). The addition of MM-398 to anti-PD-L1 also did not further increase the ratio of $CD8^+$ T cells to regulatory T cells (Tregs) (Figure 6B). These data suggest that the addition of MM-398 to anti-PD-L1 may not further increase the trafficking or proliferation of $CD8^+$ T cells in the tumor. However, $CD8^+$ T cells detected in the treatment setting of MM-398 + anti-PD-L1 were more functionally cytotoxic, as indicated by higher levels of granzyme B (Figure 6C). Taken together, these data demonstrate that Top1 inhibitors can enhance T-cell-mediated cytotoxicity

ARTICLE



Groups	Month 0	Month 50	Month 100	Month 150	Month 200
TP53INP1 low	84	16	7	2	1
TP53INP1 high	84	26	9	2	1

Figure 3. Association of TP53INP1 expression and clinical factors in melanoma patients. **A)** The Cancer Genome Atlas (TCGA) Kaplan-Meier analysis of postaccession survival in stage III cutaneous melanoma patients based on expression of TP53INP1: TP53INP1 high (above median expression, n = 84), TP53INP1 low (below median, n = 84). **B)** TCGA analysis of the association of lymphocyte score with the expression of TP53INP1 in melanoma patients: lymphocyte score low (scores 0–3, n = 167), lymphocyte score high (scores 4–6, n = 162). **C)** The association of the expression of TP53INP1 with cytolytic activity score in melanoma patients in TCGA: TP53INP1 high (above median expression, n = 219), TP53INP1 low (below median expression, n = 216). All statistical tests were two-sided. *P* values were calculated using the log-rank test **(A)** and Mann-Whitney test **(B and C)**. TCGA = The Cancer Genome Atlas; TP53INP1 = tumor protein 53–inducible nuclear protein 1.

of tumor cells and can enhance the efficacy of cancer immunotherapy.

Discussion

Checkpoint inhibitors targeting immunomodulatory factors such as CTLA-4 and PD1/-L1 have resulted in improved clinical outcomes for a variety of cancer patients. However, a common theme that remains is the large patient population that garners little to no benefit from cancer immunotherapy. For this study, we hypothesized that we could enhance the efficacy of cancer immunotherapy by developing combinatorial strategies to increase the sensitivity of melanoma tumor cells to T-cell-based cytotoxicity. We have identified Top1 inhibitors as potential agents to be used in combination with cancer immunotherapy in order to increase antitumor efficacy.

A number of recent studies have provided support for the combination of chemotherapy and immunotherapy in cancer treatment (5,20,21), and some have implicated Topoisomerase inhibitors specifically. Haggerty et al. showed that Topoisomerase inhibitors can increase the expression of melanoma tumor antigens and increase tumor recognition by T cells (22), both of which are important factors for T-cell-mediated antitumor responses. Additionally, Topotecan was shown to upregulate expression of MHC class I and interferon-β in breast cancer cells, suggesting a mechanism of chemotherapy-enhanced antitumor immunity (23).

We also found that the ability of Top1 inhibitors to increase the sensitivity of melanoma cells to T-cell-mediated killing depends on the upregulation of TP53INP1, a TP53-responsive factor that has been shown to positively regulate tumor cell apoptosis (18). TP53INP1 can mediate tumor cell apoptosis in response to TP53 activation by regulating the transcription of *P21*, *PIG3*, and *BAX* (24). In our studies, we found that overexpression of TP53INP1 enhanced T-cell-mediated killing of tumor cells. On the other hand, gene silencing of TP53INP1 had an inhibitory effect on T-cell-mediated killing of Top1 inhibitor–treated melanoma cells. These data lend further credence to the role of TP53INP1 in mediating tumor cell death in response to T-cell-based cancer immunotherapy.

Given the interplay between TP53INP1 and TP53, we interrogated our T-cell-killing phenotype in the context of TP53 and found that the effect of Top1 inhibitors on T-cell-mediated killing of melanoma was dependent on TP53. TP53 is a key tumor suppressor whose expression and function are frequently deregulated in cancer cells, providing them with a survival and development advantage (25). Our data suggest that Top1 inhibitor enhancement of T-cell-mediated killing can be affected by the mutational status of TP53. Of the eight cell lines used in our study, we observed an increase in T-cell-mediated killing following Top1 inhibitor treatment in five cell lines that are wild-type for TP53 and in 2 cell lines carrying a non-hot-spot S241F missense mutation. The S241F missense mutation is a so-called non-hot-spot mutation, as it likely retains TP53 transcriptional activity (26–28). The only cell line in which we did not observe enhancement in T-cell-mediated cytotoxicity following Top1 inhibitor treatment carries a mutation in the oligomerization domain of TP53 (F341L), which results in a nonfunctional protein (29,30). There have been a number of reports that have indicated that one important factor of tumor suppressors is their effect on the immune response (10,31,32). These studies highlight the effect of tumor suppressor genes not only on the integrity of cells, but on the integrity of immunity, and how their functions may help potentiate effective antitumor immune responses.

We also demonstrated that the combination of a Top1 inhibitor with PD1/-L1 targeting antibodies resulted in statistically significant improvements in tumor control and survival. This suggests that the addition of the Top1 inhibitor resulted in tumors being more immunogenic and therefore more responsive to T-cell-mediated antitumor immune effects. This provides the basis for clinical investigations into the use of Top1 inhibitors to augment the efficacy of cancer immunotherapy. One caveat of chemotherapy-immunotherapy combinations is the potential for lymphodepletion by chemotherapy. However, our data suggest that MM-398 was not detrimental to T-cell

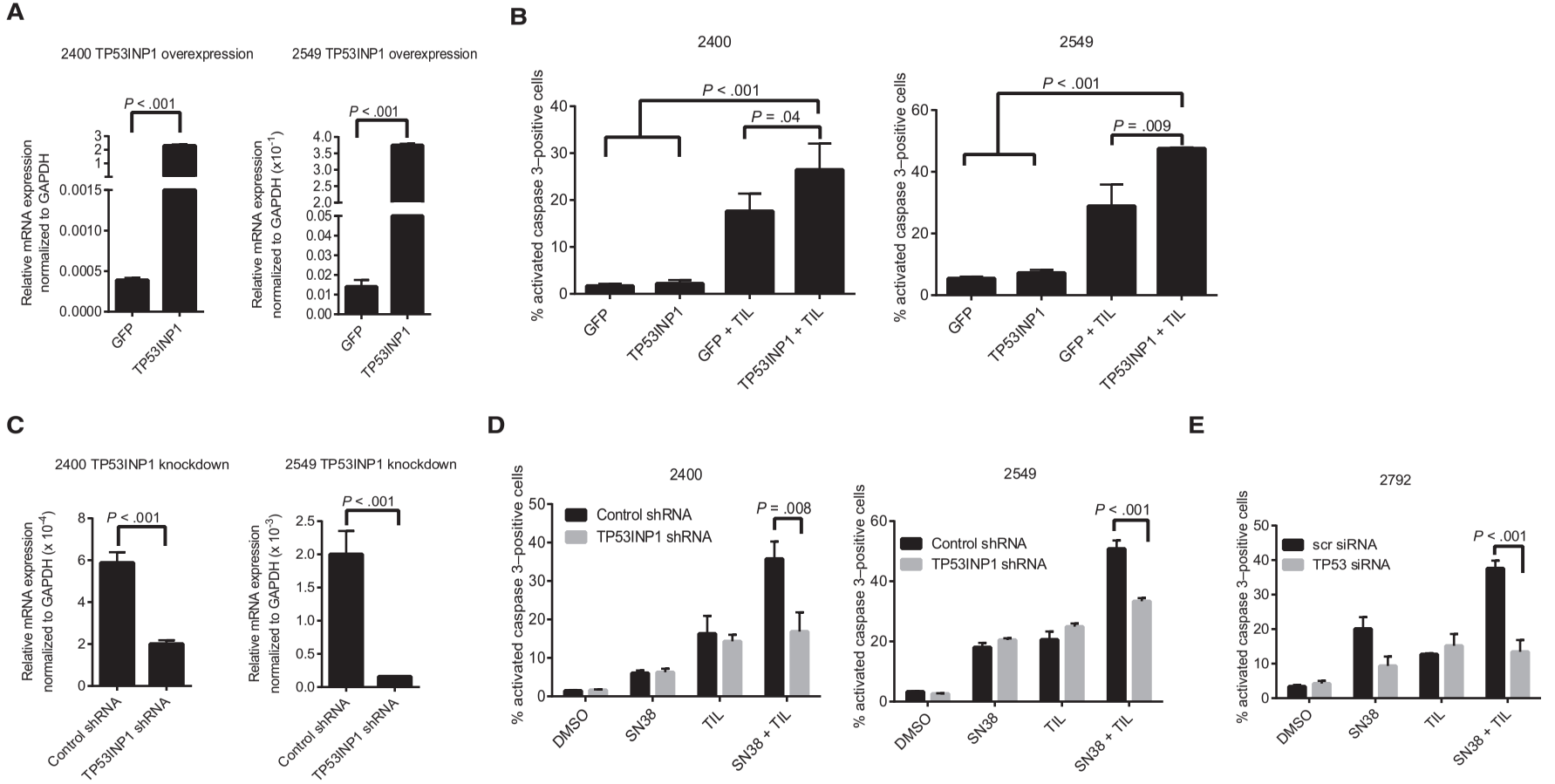
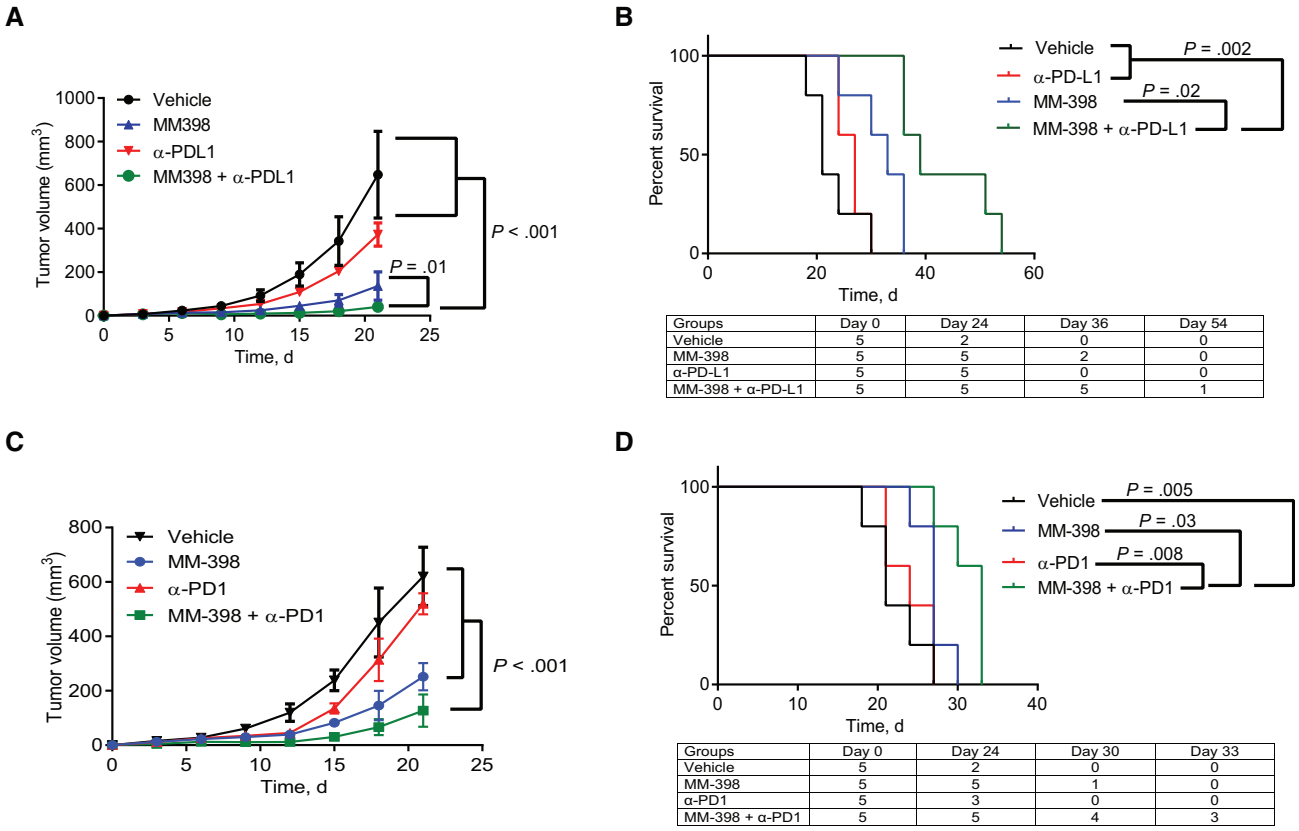


Figure 4. Effect of TP53INP1 on T-cell-mediated killing of Top1 inhibitor–treated melanoma cells. Melanoma 2400 and 2549 cells were infected with a green fluorescent protein (GFP)–tagged lentiviral vector encoding TP53INP1, or a GFP-tagged lentiviral vector as a control. **A)** Quantitative real-time polymerase chain reaction (qRT-PCR) validation of overexpression of TP53INP1 in 2400 and 2549. **B)** TP53INP1-overexpressing and GFP-overexpressing control cells were incubated with autologous TILs for three hours. Apoptosis was then quantified by flow cytometry analysis of activated caspase 3. **C)** Melanoma 2400 and 2549 cells were infected with a lentiviral vector carrying a scrambled shRNA sequence (control shRNA) or an RNAi sequence targeting TP53INP1. Stable cells were isolated after puromycin selection. qRT-PCR was used to validate reduction in the expression of TP53INP1 in 2400 and 2549. **D)** Control or TP53INP1 knockdown cells were treated with the Top1 inhibitor SN38 or DMSO for 24 hours at 1 μM. SN38-treated or control-treated cells were then processed for activated caspase 3 staining and quantification of apoptosis; or washed and then incubated with autologous tumor-infiltrating lymphocytes (TILs). Cells were then processed and stained for quantification of apoptosis. **E)** Melanoma cell line 2792 was transfected with TP53 siRNA or scrambled siRNA; 24 hours later, transfected cells were treated with SN38 at 1 μM for 24 hours, before being washed and incubated with autologous TILs. Cells were then processed and stained for activated caspase 3 for flow cytometry analysis of apoptosis. Data are all mean ± SD. Data are representative of at least two independent experiments. All statistical tests were two-sided. *P* values were calculated using the unpaired *t* test. GFP = green fluorescent protein; qRT-PCR = quantitative real time polymerase chain reaction; shRNA = small hairpin RNA; siRNA = small interfering RNA; TILs = tumor-infiltrating lymphocytes; TP53INP1 = tumor protein 53–inducible nuclear protein 1.



Groups	Day 0	Day 24	Day 36	Day 54
Vehicle	5	2	0	0
MM-398	5	5	2	0
α-PD-L1	5	5	0	0
MM-398 + α-PD-L1	5	5	5	1

Groups	Day 0	Day 24	Day 30	Day 33
Vehicle	5	2	0	0
MM-398	5	5	1	0
α-PD1	5	3	0	0
MM-398 + α-PD1	5	5	4	3

Figure 5. Antitumor effect of combining liposomal irinotecan (MM-398) with immune checkpoint blockade. **A)** C57BL/6 mice were challenged with MC38/gp100 cells. Three days later when tumors were palpable, mice were randomized into treatment groups (n = 5) receiving the Top1 inhibitor MM-398 (40 mg/kg), anti-PD-L1 antibody (150 μg), or both MM-398 and anti-PD-L1 antibody. Vehicle group received PBS and isotype-matched control antibody (150 μg). Beginning on day 3, mice received once weekly doses of MM-398 (i.v.) and antibody was administered every 3 days (i.p.). Mean tumor volume is displayed. Data are mean ± SD. **B)** Kaplan-Meier survival curves for mice treated with MM-398 and/or anti-PD-L1 antibody. **C)** Mean tumor volume in C57BL/6 mice challenged with MC38/gp100 and then treated with MM-398 alone (40 mg/kg i.v. once weekly) or in combination with anti-PD1 (200 μg i.p. every three days). Data are mean ± SD. **D)** Kaplan-Meier survival curves of treated mice. Data are representative of at least two independent experiments. All statistical tests are were two-sided. *P* values were calculated using Tukey's test **(A and C)** and log-rank test **(B and D)**. PD1 = programmed death 1; PD-L1 = programmed death-ligand 1.

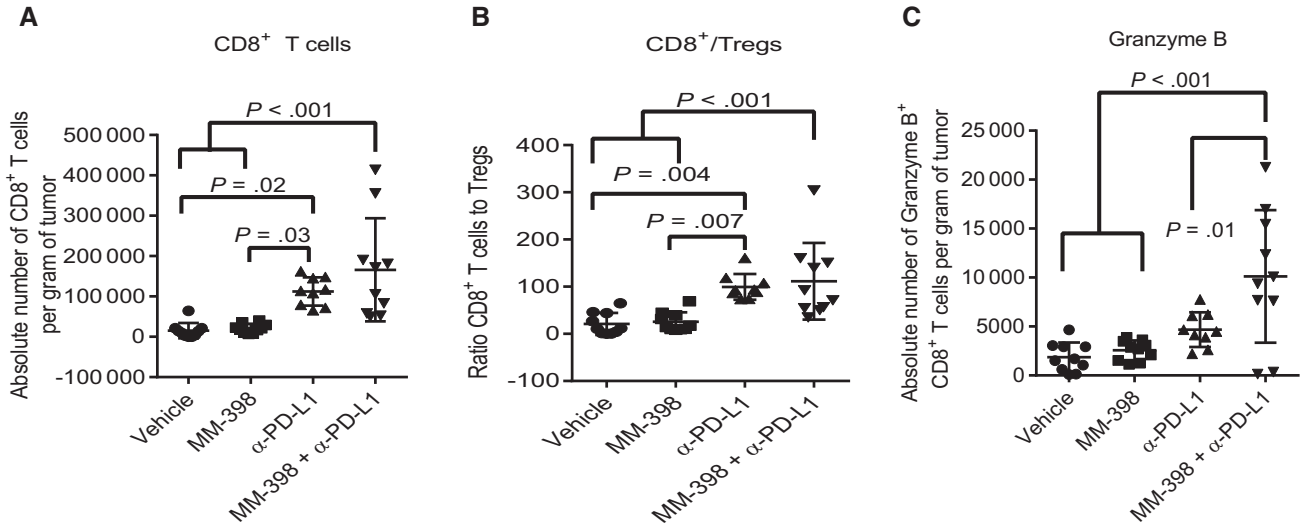


Figure 6. Ex vivo immune profiling. **A)** Analysis of the absolute number of $CD8^+$ T cells normalized to tumor weight in the four treatment groups. **B)** Ratio of $CD8^+$ T cells to regulatory T cells (Tregs) infiltrating the tumors. **C)** Quantification of $CD8^+$ T cell function by the absolute number of granzyme B^+ cells normalized to tumor weight. Data are all mean ± SD. Data are representative of at least two independent experiments. All statistical tests were two-sided. *P* values were calculated using Tukey's test.

ARTICLE

generation or tumor infiltration, as we did not detect any deficits in treatment settings involving the Top1 inhibitor. Our observation of increased antitumor activity in the combination setting suggests that the primary effect of Top1 inhibitor is on the tumor cells, thereby making them more susceptible to T-cell-induced apoptosis. Additionally, the addition of MM-398 to anti-PD-L1 saw a statistically significant increase in the cytotoxic capacity of $CD8^+$ T cells, suggesting that this Top1 inhibitor may be remodeling the tumor microenvironment to allow for more pervasive cytotoxicity by effector T cells.

Despite the safety of MM-398 + anti-PD1/-L1 in preclinical experiments, one potential limitation of our study is that unexpected toxicities may emerge in early clinical testing. Our in vivo studies involved concurrent dosing of MM-398 and anti-PD1/-L1 antibody. However, this may not be the most optimal dosing regimen, and further development of this combination for clinical application should include additional investigation into the dosing regimen, because priming or a run-in with the Top1 inhibitor prior to introduction of the immune checkpoint blocker may lead to additional antitumor benefits. Additionally, our studies focused only on the ultimate effector function of T cells, namely the lysis of tumor cells. However, other steps in the cancer immunity cycle (33), such as priming and activation of antigen presentation cells may also be influenced by Top1 inhibitors.

In summary, Top1 inhibitors can augment T-cell-mediated antitumor immune responses and enhance the efficacy of cancer immunotherapy. The underlying mechanism of this combination is based on the TP53-responsive protein TP53INP1; suggesting that the tumor suppressor TP53 plays an integral role in the antitumor immune response and that TP53 status may be an important consideration in the potential selection of cancer patients for this combinatorial treatment strategy.

Funding

This work was supported by the the National Institutes of Health (R01CA187076 and 5R01CA184845-04 to PH, P50CA083639 and UH3TR000943 to AS, P50CA093459 to JAM, and P30CA016672 to the Flow Cytometry Core Facility); Ovarian Cancer Research Fund; Foundation for Women's Cancer; American Cancer Society Research Professor Award to AS; and by philanthropic contributions to the MD Anderson Cancer Center (MDACC) Melanoma Moon Shots Program; a Melanoma Research Alliance Team Science Award; the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation; the Talla Family Revocable Trust; the Aim at Melanoma Foundation; Miriam and Jim Mulva Research Fund; Jurgen Sager and Transocean Melanoma Research Fund; the El Paso Foundation for Melanoma Research; and also by the Cancer Prevention and Research Institute of Texas (CPRIT; RP170401 to PH; RP140106 and RP170067 to JAM).

Notes

Authors: Jodi A. McKenzie, Rina M. Mbofung*, Shruti Malu*, Min Zhang, Emily Ashkin, Seram Devi, Leila Williams, Trang Tieu, Weiyi Peng, Sunila Pradeep, Chunyu Xu, Soraya Zorro Manrique, Chengwen Liu, Lu Huang, Yuan Chen, Marie-Andree Forget, Cara Haymaker, Chantale Bernatchez, Nikunj Satani, Florian Muller, Jason Roszik, Ashish Kalra, Timothy Heffernan, Anil Sood, Jianhua Hu, Rodabe Amaria, R. Eric Davis, Patrick Hwu

Affiliations of authors: Department of Melanoma Medical Oncology (JAM, RMM, SM, EA, LW, WP, CX, SZM, CL, LH, YC, MAF, CH, CB, JR, RA, PH), Department of Lymphoma/Myeloma (MZ, RED), Department of Sarcoma Medical Oncology (SD), Institute for Applied Cancer Science (TT, TH), Department of Gynecologic Oncology and Reproductive Medicine (SP, AS), Department of Cancer Imaging Systems (NS, FM), Center for RNA Interference and Non-coding RNA (AS), and Department of Biostatistics (JH), The University of Texas MD Anderson Cancer Center, Houston, TX; Merrimack Pharmaceuticals, Cambridge, MA (AK).

The funders had no role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

The authors declare no competing financial interests.

Author contributions: JAM, RM, SM, and PH designed and executed studies. JAM, RM, SM, SD, EA, CX, SZM, CL, LW, LH, YC, SP, and WP performed experiments. JAM, RM, MZ, WP, JR, RED, JH, and PH analyzed and interpreted data. JAM wrote the manuscript. JAM, RED, WP, LH, RA, and PH edited the manuscript. TT, TH, CB, MF, CH, FM, NS, WP, RA, AS, and AK provided key reagents.

Microarray data supporting the findings of this study have been deposited in GEO repository with the accession code GSE100317.

The authors would like to thank past and present members of the MDACC TIL lab for tumor/TIL processing and banking.

References

1. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363(8):711–723.
2. Brahmer JR, Drake CG, Wollner I, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: Safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010; 28(19):3167–3175.
3. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366(26): 2443–2454.
4. Ribas A, Puzanov I, Dummer R, et al. Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): A randomised, controlled, phase 2 trial. *Lancet Oncol*. 2015;16(8): 908–918.
5. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N J Engl Med*. 2011;364(26): 2517–2526.
6. Topalian SL, Sznol M, McDermott DF, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol*. 2014;32(10):1020–1030.
7. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med*. 2015;373(2): 123–135.
8. Motzer RJ, Escudier B, McDermott DF, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med*. 2015;373(19):1803–1813.
9. Kelderman S, Schumacher TN, Haanen JB. Acquired and intrinsic resistance in cancer immunotherapy. *Mol Oncol*. 2014;8(6):1132–1139.
10. Peng W, Chen JQ, Liu C, et al. Loss of PTEN promotes resistance to T cell-mediated immunotherapy. *Cancer Discov*. 2016;6(2):202–216.
11. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature*. 2015;523(7559):231–235.
12. Zaretsky JM, Garcia-Diaz A, Shin DS, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *N Engl J Med*. 2016;375(9): 819–829.
13. Chacon JA, Wu RC, Sukhumalchandra P, et al. Co-stimulation through 4-1BB/CD137 improves the expansion and function of CD8(+) melanoma tumor-infiltrating lymphocytes for adoptive T-cell therapy. *PloS One*. 2013;8(4): e60031.
14. Peng W, Ye Y, Rabinovich BA, et al. Transduction of tumor-specific T cells with CXCR2 chemokine receptor improves migration to tumor and antitumor immune responses. *Clin Cancer Res*. 2010;16(22):5458–5468.
15. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. 2010;70(2):440–446.

16. Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. *Cell*. 2015;161(7):1681–1696.
17. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*. 2015;160(1–2):48–61.
18. Okamura S, Arakawa H, Tanaka T, et al. p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis. *Mol Cell*. 2001;8(1):85–94.
19. Kalra AV, Kim J, Klinz SG, et al. Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor prodrug conversion. *Cancer Res*. 2014;74(23):7003–7013.
20. Lynch TJ, Bondarenko I, Luft A, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: Results from a randomized, double-blind, multicenter phase II study. *J Clin Oncol*. 2012;30(17):2046–2054.
21. Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res*. 2003;63(15):4490–4496.
22. Haggerty TJ, Dunn IS, Rose LB, et al. Topoisomerase inhibitors modulate expression of melanocytic antigens and enhance T cell recognition of tumor cells. *Cancer Immunol Immunother*. 2011;60(1):133–144.
23. Wan S, Pestka S, Jubin RG, Lyu YL, Tsai YC, Liu LF. Chemotherapeutics and radiation stimulate MHC class I expression through elevated interferon-beta signaling in breast cancer cells. *PloS One*. 2012;7(3):e32542.
24. Tomasini R, Samir AA, Carrier A, et al. TP53INP1s and homeodomain-interacting protein kinase-2 (HIPK2) are partners in regulating p53 activity. *J Biol Chem*. 2003;278(39):37722–37729.
25. Muller PA, Vousden KH. Mutant p53 in cancer: New functions and therapeutic opportunities. *Cancer Cell*. 2014;25(3):304–317.
26. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: Origins, consequences, and clinical use. *Cold Spring Harbor Perspect Biol*. 2010;2(1):a001008.
27. Muller PA, Vousden KH. p53 mutations in cancer. *Nat Cell Biol*. 2013;15(1):2–8.
28. Kovvali GK, Mehta B, Epstein CB, Lutzker SG. Identification of partial loss of function p53 gene mutations utilizing a yeast-based functional assay. *Nucleic Acids Res*. 2001;29(5):E28.
29. Kawaguchi T, Kato S, Otsuka K, et al. The relationship among p53 oligomer formation, structure and transcriptional activity using a comprehensive missense mutation library. *Oncogene*. 2005;24(46):6976–6981.
30. Freed-Pastor WA, Prives C. Mutant p53: One name, many proteins. *Genes Devel*. 2012;26(12):1268–1286.
31. Hutcheson J, Witkiewicz AK, Knudsen ES. The RB tumor suppressor at the intersection of proliferation and immunity: Relevance to disease immune evasion and immunotherapy. *Cell Cycle*. 2015;14(24):3812–3819.
32. Cortez MA, Ivan C, Valdecanas D, et al. PDL1 Regulation by p53 via miR-34. *J Natl Cancer Inst*. 2016;108(1):djv303.
33. Chen DS, Mellman I. Oncology meets immunology: The cancer-immunity cycle. *Immunity*. 2013;39(1):1–10.