iScience, Volume *24*

Supplemental Information

Sensitization of ovarian tumor to immune checkpoint blockade by boosting senescence-associated secretory phenotype

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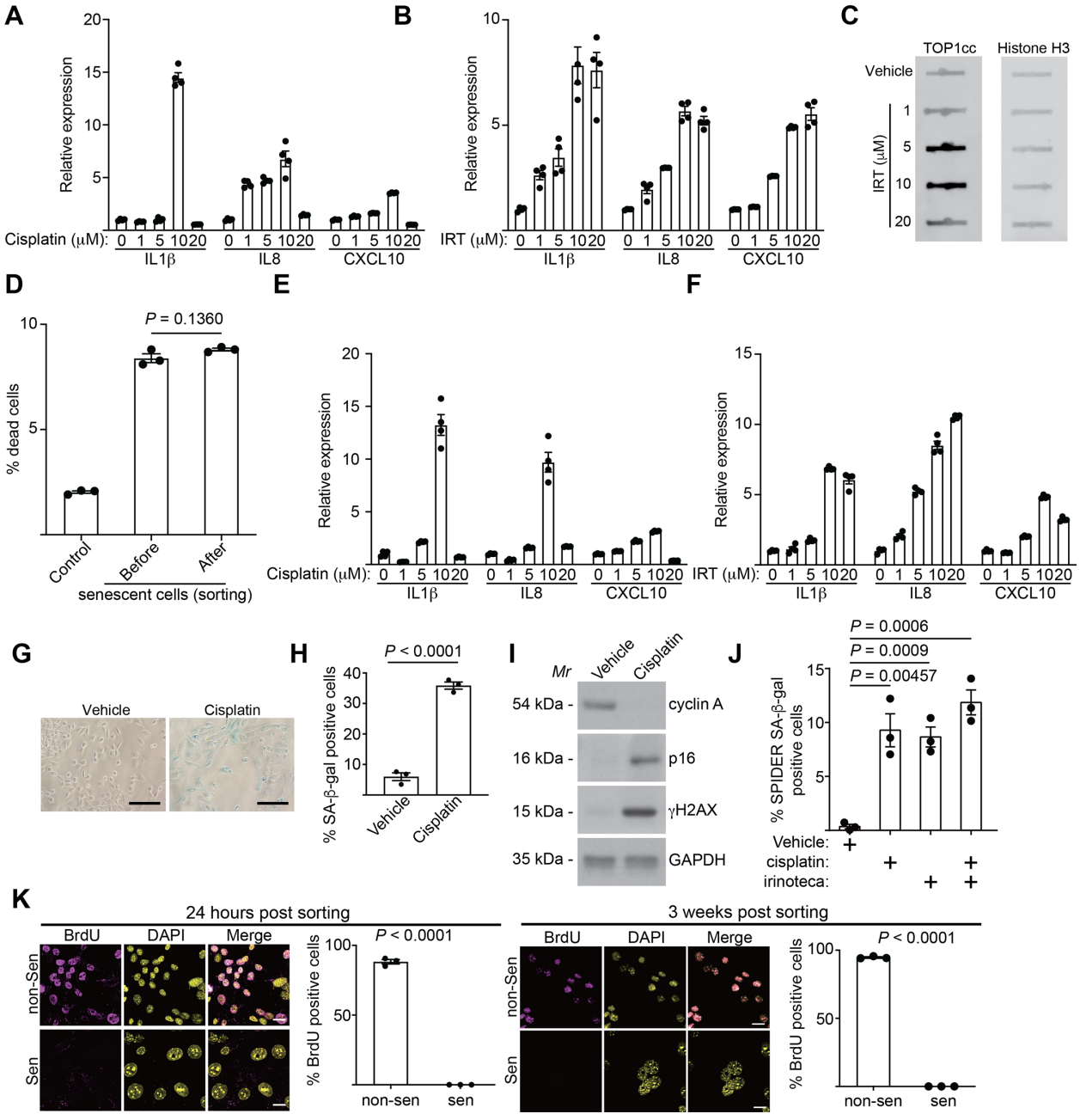
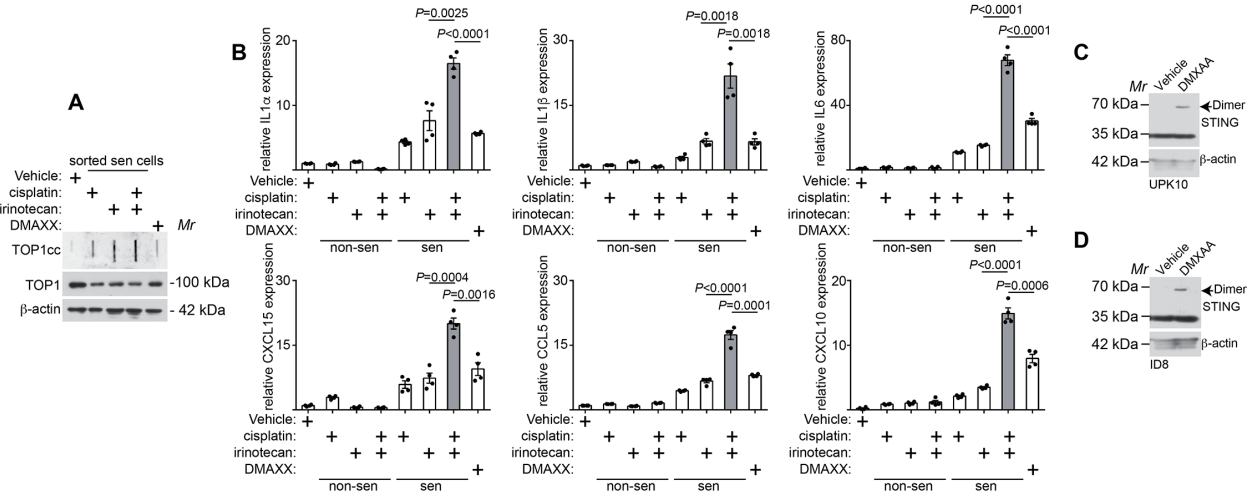


Supplemental Figure 1: Isolation of SASP-boosted therapy-induced senescent cells, related to Figure 1.

(A-C) UPK10 cells were treated with the indicated concentration of cisplatin (**A**) or irinotecan (**B-C**) for three days. After three days of release, expression of the indicated SASP factors was examined by qRT-PCR (**A-B**). Level of TOP1cc in the irinotecan-treated cells was examined by slot blot (**C**). (n=4 biologically independent experiments).

(**D**) Percentage of dead cells in senescent UPK10 cells induced by a combination of 10 μM cisplatin and 10 μM irinotecan before and after flow cytometry sorting. (n=3 biologically independent experiments).

(**E-F**) ID8 cells were treated with the indicated concentration of cisplatin (**E**) or irinotecan (**F**) for three days. After three days of release, expression of the indicated SASP factor was examined by qRT-PCR. (n=4 biologically independent experiments).
(**G-I**) ID8 cells were treated with 10μM cisplatin for three days and released for three days. SA-β-gal activity was examined (**G**) and quantified (**H**). Expression of the indicated proteins were examined by immunoblot in the indicated cells (**I**).
(**J**) ID8 cells were treated with 10μM cisplatin, 10μM irinotecan or a combination for three days and released for three days. SA-β-gal positive cells were quantified using SPiDER SA-β-gal assay by flow cytometry.
(K) Sorted senescent and non-senescent cells from cisplatin and irinotecan treated ID8 cells at the indicated time points post sorting (24 hrs or 3 weeks) were labeled with BrdU for 24 hrs and BrdU incorporation was examined by immunofluorescence staining and quantified.
(n=3 biologically independent experiments).
Data represent mean ± SEM. Scale bar = 100 μm in S1G and 20 μm in S1K. *P* values were calculated using a two-tailed *t*-test.

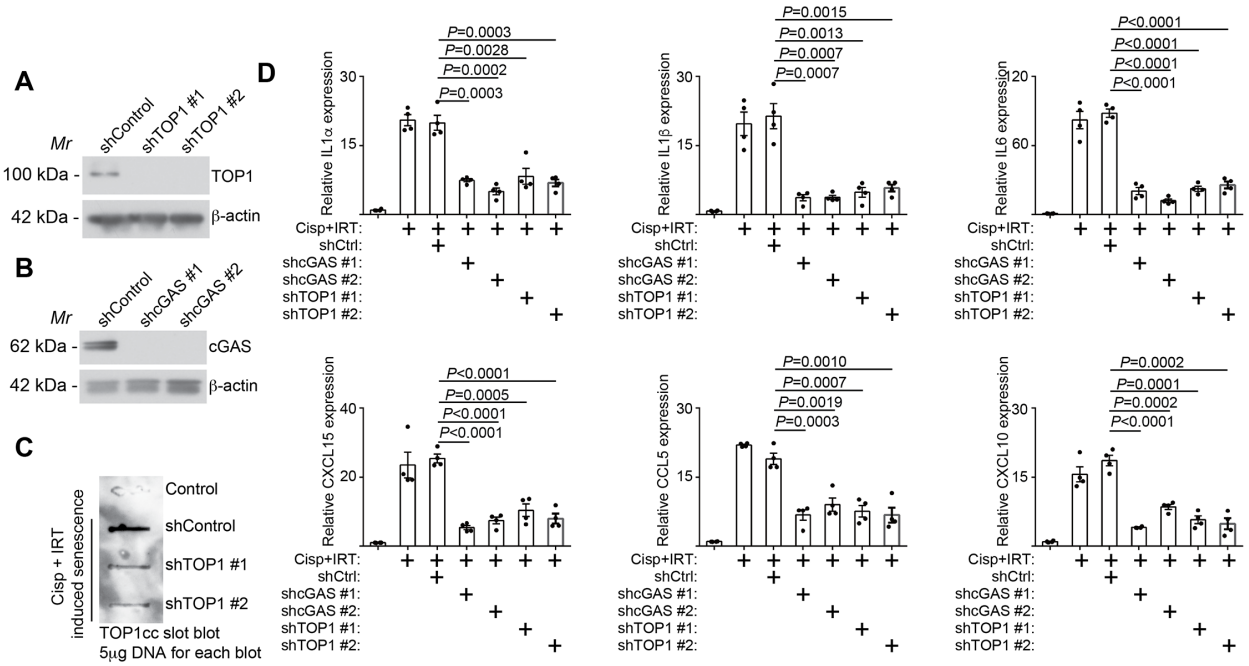


Supplemental Figure 2: TOP1 inhibitor irinotecan boosts SASP expression in cisplatin-induced ID8 senescent cells, related to Figure 2.

(A-B) ID8 cells were treated with 10μM cisplatin, 10μM irinotecan, a combination, or 10 μM DMXAA for three days and released for three days. Expression of TOP1cc, TOP1 and a loading control β-actin examined by immunoblot in the indicated cells **(A)**. Expression of the indicated SASP factors in sorted senescent and non-senescent ID8 cells from the indicated treatment was determined by qRT-PCR (n=3 biologically independent experiments) **(B)**.

(C-D) STING dimerization induced by DMXAA treatment was determined by immunoblot in UPK10 (**C**) and ID8 (**D**) cells.

Data represent mean ± SEM. *P* values were calculated using a two-tailed *t*-test.



Supplemental Figure 3: Irinotecan-boosted SASP in senescent ID8 cells depends on cGAS and TOP1, related to Figure 3.

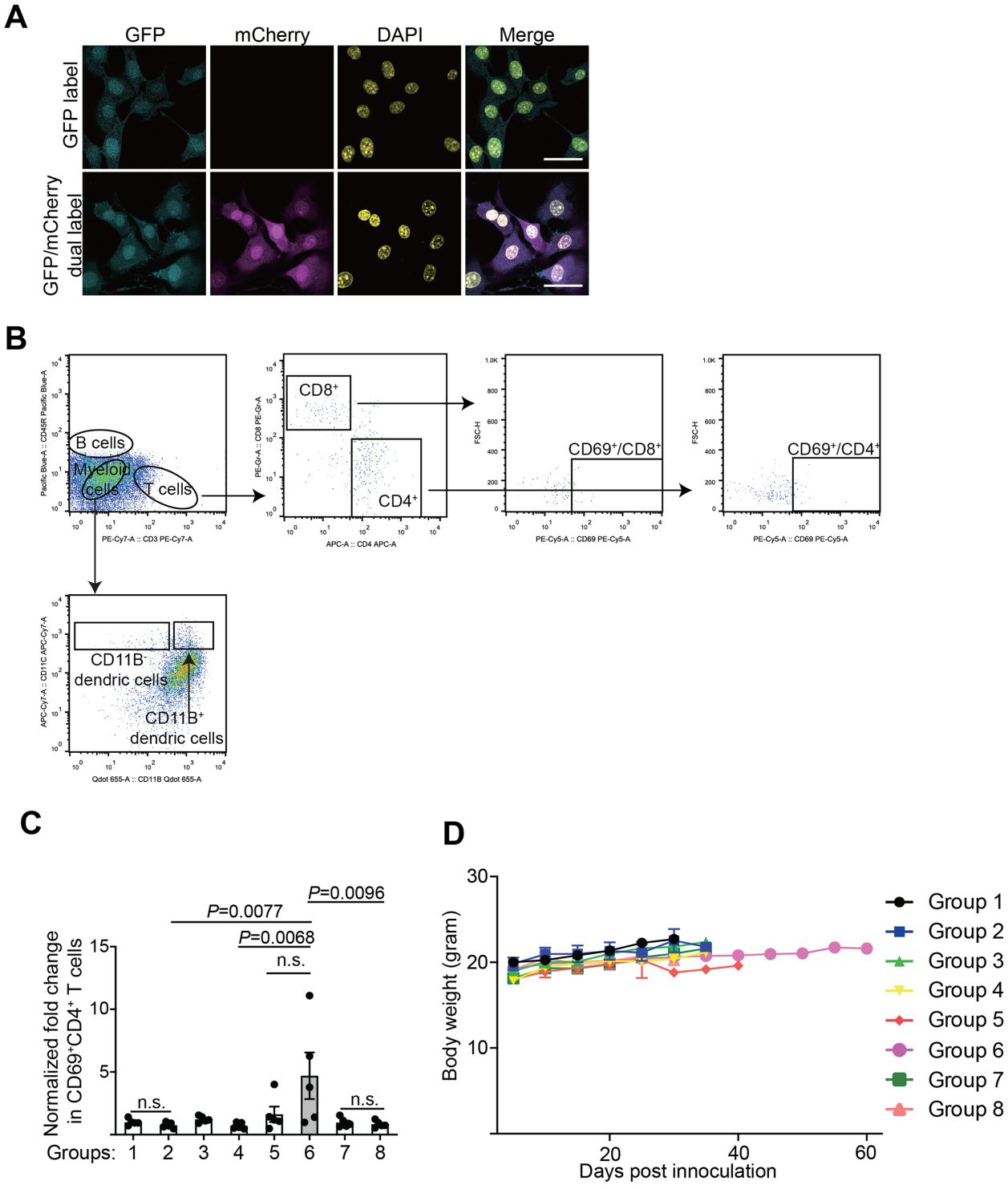
(A) Expression of TOP1 and a loading control β-actin in ID8 cells expressing the indicated shTOP1s or a shControl was determined by immunoblot.

(B) Expression of cGAS and a loading control β-actin in ID8 cells expressing the indicated shcGASs or a shControl was determined by immunoblot.

(C) Expression of TOP1cc in ID8 cells expressing the indicated shTOP1s or a shControl was determined by slot blot.

(D) ID8 cells were treated with 10μM cisplatin, 10μM irinotecan or a combination for three days and released for three days. Expression of the indicated SASP factors in the sorted indicated non-senescent and senescent cells was determined by qRT-PCR (n=3 biologically independent experiments).

Data represent mean ± SEM. *P* values were calculated using a two-tailed *t*-test.



Supplemental Figure 4: Adoptive transfer of SASP-boosted senescent cells does not display overt toxicity, related to Figure 4.

(A) Confirmation of GFP and mCherry expression in UPK10 cells used for generating pre-established tumors and adoptive transfer. GFP positive cells were used to generate orthotopic tumors, and GFP and mCherry double positive cells were used for senescence induction and subsequent transfer.

(**B**) The gating strategy used in the present study.
(C) Fold changes in percentage of $CD69^+/CD4^+$ T cells in $CD4^+$ T cell population (normalized by tumor weight) were determined in tumors dissected from the indicated treatment groups (n = 5 biologically independent mice per group).
(D) Body weight of mice from the indicated treatment groups during the entire experimental period (n = 5 biologically independent mice per group).
Data represent mean ± SEM. Scale bar = 20 μm in S4A. *P*-values were calculated using multiple *t* test. n.s.: not significant

Supplemental Table 1: The oligonucleotides used for quantitative RT-PCR, related to Figure 2.

Name	Sequence	Application
Mouse *IL1α* forward	5'-CCAGAAGAAAATGAGGTCGG-3'	RT-qPCR
Mouse *IL1α* reverse	5'-AGCGCTCAAGGAGAAGACC-3'	RT-qPCR
Mouse *IL1β* forward	5'-TGTGCAAGTGTCTGAAGCAGC-3'	RT-qPCR
Mouse *IL1β* reverse	5'-TGGAAGCAGCCCTTCATCTT-3'	RT-qPCR
Mouse *IL6* forward	5'-GCTACCAAACTGGATATAATCAGGA-3'	RT-qPCR
Mouse *IL6* reverse	5'-CCAGGTAGCTATGGTACTCCAGAA-3'	RT-qPCR
Mouse *CXCL15* forward	5'-AGAGGCTTTTCATGCTCAACA-3'	RT-qPCR
Mouse *CXCL15* reverse	5'-CCATGGGTGAAGGCTACTGT-3'	RT-qPCR
Mouse *CCL5* forward	5'-CCACTTCTTCTCTGGGTTGG-3'	RT-qPCR
Mouse *CCL5* reverse	5'-GTGCCCACGTCAAGGAGTAT-3'	RT-qPCR
Mouse *CXCL10* forward	5'-TCAGCACCATGAACCCAAG-3'	RT-qPCR
Mouse *CXCL1*0 reverse	5'-CTATGGCCCTCATTCTCACTG-3'	RT-qPCR
Mouse *B2M* forward	5'-AGTTAAGCATGCCAGTATGGCCGA-3'	RT-qPCR
Mouse *B2M* reverse	5'-ACATTGCTATTTCTTTCTGCGTGC-3'	RT-qPCR

Transparent Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-BrdU (BU1/75 (ICR1))	Novus	Cat# NB500-169
Rabbit polyclonal anti-TOP1	Proteintech	Cat# 20705-1-AP
Mouse monoclonal anti-Topoisomerase I-DNA Covalent Complexes (TOP1cc) (clone 1.1A)	Millipore	Cat# MABE1084
Mouse monoclonal anti-cGAS (D9)	Santa Cruz	Cat# sc-515777
Rabbit monoclonal anti-STING (D2P2F)	Cell Signaling Technology	Cat# 13647 S
Rabbit polyclonal anti-Cyclin A (H432)	Santa Cruz	Cat# sc-751
Mouse monoclonal anti-β-actin	Sigma	Cat# A2228
650™ anti-mouse/human CD11b	Biolegend	Cat# 101259
APC/Cyanine7 anti-mouse CD11c	Biolegend	Cat# 117324
APC anti-mouse CD4	Biolegend	Cat# 100516
PE anti-mouse CD8a	Biolegend	Cat# 100708
PE/Cy5 anti-mouse CD69	Biolegend	Cat# 104510
Anti-PD-1 antibody (clone 29F.1A12)	Bio X Cell	Cat# BE0273
Mouse anti-CDKN2A/p16INK4a antibody (DSC50.1)	Abcam	Cat# ab1623
Rabbit anti-gamma H2A.X (phospho S139) antibody [EP854(2)Y]	Abcam	Cat# ab81299

Mouse anti-p21(187)	Santa Cruz	Cat# sc-817
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit monoclonal antibody	Cell Signaling Technology	Cat# 3033
NF-κB p65 (D14E12) XP® Rabbit monoclonal antibody	Cell Signaling Technology	Cat# 8242
Phospho-p38 MAPK (Thr180/Tyr182) Antibody	Cell Signaling Technology	Cat# 9211
p38α MAPK (L53F8) Mouse monoclonal antibody	Cell Signaling Technology	Cat# 9228
Histone H3 (1B1B2) Mouse monoclonal antibody	Cell Signaling Technology	Cat# 14269
Chemicals, Peptides, and Recombinant Proteins		
Cisplatin	Selleck	Cat# S1166
Irinotecan	Selleck	Cat# S2217
5,6-Dimethylxanthenone-4-acetic Acid (DMXAA)	Sigma	Cat# D5817
SPiDER- β Gal	Dojindo Molecular Technologies	Cat# SG02-10
5-Bromo-2'-deoxyuridine (BrdU)	Sigma	Cat# B5002
4' 6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma	Cat# D9542
Paraformaldehyde	Sigma	Cat# 158127
Collagenase	Sigma	Cat# C5138
Hyaluronidase	Sigma	Cat# H3884
DNase 1	Sigma	Cat# D5025
Lipofectamine 2000	Thermo Fisher	Cat# 11668019
Hydrochloric acid	Fisher chemical	Cat# SA55
Experimental Models: Cell Lines		
UPK10 ovarian cancer cells		N/A
ID8 ovarian cancer cells		N/A
Lentivirus vectors		
pCMV-GFP	Addgene	Cat# 11153
pLV-mCherry	Addgene	Cat# 36084
pLKO.1-shTOP1 #1(TRCN0000011883)	Wistar Facility	N/A
pLKO.1-shTOP1 #2 (TRCN0000011886)	Wistar Facility	N/A
pLKO.1-shcGAS #1(TRCN0000416658)	Wistar Facility	N/A
pLKO.1-shcGAS #2 (TRCN0000421958)	Wistar Facility	N/A
Critical Commercial Assays		
ACK Lysis Buffer	Thermo Fisher	Cat# A1049201
Live/Dead Fixable Aqua Dead Cell Stain Kit	Thermo Fisher	Cat# L34965
eBioscience fixation/ permeabilization kit	Thermo Fisher	Cat# 88-8824-00
LookOut Mycoplasma polymerase chain reaction (PCR) detection kit	Sigma	Cat# MP0035
Human Topoisomerase 1 ICE Assay Kit	TopoGEN	Cat# TG1020-1
Mouse Cytokine Array C1 kit	RayBiotech	Cat# AAM-CYT-1-2
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher	Cat# 34580

ViraPower kit	Invitrogen	Cat# A11141
Oligonucleotides		
See Table S1		

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Rugang Zhang (rzhang@wistar.org)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

The mouse ovarian cancer cell lines UPK10 and ID8 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cell lines are authenticated at The Wistar Institute's Genomics Facility using short tandem repeat DNA profiling. Regular mycoplasma testing was performed using the LookOut Mycoplasma polymerase chain reaction (PCR) detection (Sigma, Cat. No: MP0035).

Mice

The protocols were approved by the Wistar Institutional Animal Care and Use Committee (IACUC). Mice are housed in solid bottom, single use ventilated or static cages. Cage bottoms and bedding are changed every two weeks for ventilated cages, and weekly for static. Lids and feeders are changed every 4 weeks. Animal quarters are serviced by individual animal caretakers who are trained to recognize the symptoms characteristic of sick animals. Each day the caretakers initial a checklist posted in each room indicating that observations were made. Temperature and humidity are monitored and documented. Staff monitors for and documents any animal welfare conditions and removes any dead animals if observed and notifies research staff and facility management. If an animal welfare condition is observed both the veterinarian and animal facility director or supervisor are notified. Animals are treated if there are open veterinary cases including weekends and holidays. 6-8-week old female C57BL/6 mouse from CRL/NCI were used for all *in vivo* experiments.

METHOD DETAILS

Lentivirus infection and sorting of GFP or mCherry labelled cells

Lentivirus was produced using the ViraPower kit (Invitrogen) based on manufacturer's instructions in the 293FT human embryonal kidney cell line by Lipofectamine 2000 transfection (Thermo Fisher. Cat. No: 11668019). Lentivirus was harvested and filtered with 0.45 μm filter 48 hrs post transfection. Cells infected with lentiviruses were selected in 1 μg/ml puromycin 48 hrs post infection. GFP or mCherry labelled cells were sorted using flow cytometry.

Senescence induction and sorting of senescent cells

UPK10 and ID8 cells were treated with 10 μM Cisplatin, 10 μM Irinotecan, or a combination for three days. The drugs were then released from drug treatment and cultured for three days or extended period as indicated. The senescent cells were labelled with SPiDER-β Gal Cellular Senescence Detection Kit (Dojindo, Cat. No: SG02-10) following the manufacture's instruction. Both senescent and non-senescent cells were sorted using flow cytometry.

BrdU incorporation assay and Immunofluorescence
Cells were plated on coverslips and labelled with 10 μg/ml BrdU for 24 hrs. Cells were fixed with 4% paraformaldehyde (PFA) for 15 mins at room temperature followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. Cells were incubated in 2.5 M hydrochloric acid at 4°C for 24 hrs. After blocking with 1% BSA in PBS, cells were incubated with primary antibody overnight at 4°C and Alexa-Fluor conjugated secondary antibody (Life Technologies) for one hr. Fluorescent images were captured using Leica TCS SP5 II scanning confocal microscope.

Immunoblot
Cells were lysed in 1X sample buffer [2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.01% bromophenol blue, 62.5 mM Tris, pH 6.8, and 0.1 M DTT] and heated to 95 °C for 10 mins. Protein concentrations were determined using the protein assay dye (Bio-Rad, Cat. No: #5000006) and Nanodrop. An equal amount of total protein was resolved using SDS polyacrylamide gel electrophoresis gels and transferred to PVDF membranes at 110 V for 2 hrs at 4 °C. Membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 hr at room temperature. Membranes were incubated overnight at 4 °C in the primary antibodies in 4% BSA/TBS + 0.025% sodium azide. Membranes were then washed four times in TBS-T for 5 min at room temperature, after which they were incubated with Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 hr at room temperature. After washing four times in TBS-T for 5 min at room temperature, proteins were visualized on film after incubation with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

TOP1 ICE (*In vivo* Complex of Enzyme) Assay and slot blot
Human Topoisomerase 1 ICE Assay Kit (TopoGEN. Cat. No:TG1020-1) was used to isolate protein-DNA samples which contain TOP1-DNA covalent complex (TOP1cc) for slot blot analysis. The isolation was performed following the manufacturer's guidelines. $5x10^5$ cells were used for ICE assay and TOP1cc analysis. Briefly, cells were lysed with 300 μL of room temperature buffer A, and then 115 μL buffer B was added to precipitate DNA. After washing with buffer C, DNA was dissolved in buffer D and buffer E. The DNA samples were kept in 37 °C to promote the recovery. Nano-Drop was used to measure the DNA concentration. 5 μg DNA was used for each slot blot analysis. Bio-Dot SF Microfiltration Apparatus (Bio Rad. Cat. No:1706542) was used for slot blot.

Quantification PCR with reverse transcription
Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instruction. Extracted RNAs were used for reverse-transcriptase PCR (RT-PCR) with High-Capacity cDNA Reverse Transcription Kit (Thermo fisher, Cat. No: 4368814). Quantitative PCR (qPCR) was performed using iTaq™ Universal SYBR® Green Supermix (BIO-RAD, Cat. No: 1725121) and QuantStudio 3 Real-Time PCR System. The oligonucleotides used for qPCR analysis were included in **Supplemental Table 1**.

Antibody array
Mouse Cytokine Array C1 kit (RayBiotech. Cat. No: AAM-CYT-1-2) was used for cytokine analysis following the manufacturer's guidelines. Briefly, cells were washed once and cultured in serum-free medium for 48 hrs. Conditioned medium was filtered (0.2 μm) and then subjected to cytokine-array analysis. After collection of conditional media, the cell number of each sample was counted. The intensities of array dots were visualized on film after incubation with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific. Cat. No:

34580). The integrated density was measured using Image J and normalized to the cell number from which the conditioned medium was generated.

In vivo mouse model and profiling of infiltrated immune cells

The protocols were approved by the Institutional Animal Care and Use Committee of the Wistar Institute. 1×10^6 UPK10 cells were unilaterally injected into the ovarian bursa sac of C57BL/6 mouse (female, 6–8 weeks old, CRL/NCI). The orthotopically transplanted cells were allowed to form tumor for 15 days. Tumor-bearing mice were randomly assigned to different treatment groups. The mice were treated for two weeks. Specifically, the mice were pre-treated by i.p. injection (1×10^6 cells per mouse) with control UPK10 cells (group 3), or senescent UPK10 cells sorted from cisplatin, irinotecan and cisplatin/irinotecan combination treated groups (group 4, 5 and 6), or 10 mg/kg DMXAA (group 8), or DMSO vehicle control (group 7). 24 hrs following the pre-treatment, the mice were treated by i.p. injection with anti-PD-1 antibody (Bio X Cell, Cat. No: BE0273, clone 29F.1A12, 10 mg/kg) or an isotype matched IgG control every 3 days.

After two weeks of treatment, the tumors were collected and digested using mixture of 10mg/mL Collagenase (Sigma, Cat No: C5138), 1 mg/mL Hyaluronidase (Sigma, Cat No: H3884) and 200 mg/mL DNase 1 (Sigma, Cat No: D5025) at 37°C for 1 hr. Single-cell suspensions were prepared, and red blood cells were lysed using ACK Lysis Buffer (Thermo Fisher, Cat No: A1049201). Live/dead cell discrimination was performed using LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher, Cat No: L34968). Cell surface staining was done for 30 min at 4°C. All data acquisition was done using an LSR II (BD) or FACSCalibur (BD) and analyzed using FlowJo software (TreeStar) or the FlowCore package in the R language and environment for statistical computing. For survival analysis, the Wistar Institute IACUC guideline was followed in determining the time for ending the survival experiments (mice succumbed to the disease or tumor burden exceeds 10% of body weight).

Immunofluorescence staining for tumor tissue sections

Formalin-fixed, paraffin-embedded tumors were sectioned, and slides were deparaffinized and rehydrated. Antigen retrieval was performed by boiling for 40 mins in citrate buffer, pH6.0 (Thermo Fisher). Endogenous peroxidases were quenched with 3% hydrogen peroxide in methanol. Sections were then blocked with 5% BSA/PBS at room temperature for 1 hr. Sections were incubated with primary mouse anti-GFP (Santa Cruz, 1:400 dilution) or rabbit anti-mCherry (Proteintech, 1:200 dilution) antibodies at 4°C overnight. Detection was performed using secondary Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher, 1:1000 dilution) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Thermo Fisher, 1:1000 dilution) at room temperature for 1 hr. The sections were counter stained with DAPI containing Duolink® in Situ mounting medium (Sigma Aldrich) and sealed. Samples were imaged on Leica TCS SP5 II Scanning Confocal Microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results are representative of a minimum of three independent experiments. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad). The Student's t-test was performed to determine *P* values of the raw data unless otherwise stated, where $P < 0.05$ was considered significant. Animal experiments were randomized. There was no exclusion from the experiments.