Supplementary information

Senescent cancer cell vaccines induce cytotoxic T cell responses targeting primary tumors and disseminated tumor cells

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Supplementary methods

Fig. S1 to S20

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Supplementary methods

Senescence induction and SA-β-Gal assay

To induce cellular senescence, cells were seeded at 2 × 10^4/mL in plates and treated with etoposide (2 μM), GSK461364 (5 μM), irradiation (IR) (12 Gy), or IR + veliparib (12 Gy + 20 μM). For IR + veliparib, the veliparib was added 1 h prior to IR. Cells treated with DMSO vehicle or veliparib alone (20 μM) served as controls. After 5 days of culture without changing the media, cells were fixed with 2% PFA. For SA-β-Gal assay, cells were incubated for 16 to 32 h at 37 °C in staining buffer containing 1 mg/mL X-Gal (X4281C, Golden Bio), 40 mM citric acid/sodium phosphate, 150 mM NaCl, 2 mM $MgCl_2$, 3.3 mM $K_3[Fe(CN)_6]$, 3.3 mM $K_4[Fe(CN)_6]$, pH = 6. After staining, images were captured on a Zeiss Axiovert 200M microscope with a 20× Plan-NeoFluar objective and Axiocam digital camera. SA-β-Gal-positive and -negative cells were counted in more than 5 fields, yielding an average percentage indicated on each SA-β-Gal image as mean ± SD. Two or more replicates were performed.

Characterization of senescence-associated secretory phenotype (SASP)

WT or STING KO CT26 cells were treated for 4 days with DMSO control, etoposide (2 μM), GSK461364 (5 μM), IR (12 Gy), or IR + veliparib (12 Gy + 20 μM), then washed with PBS and cultured continuously for 2 days in fresh medium in order to collect conditioned media containing senescence-associated secretory phenotype (SASP) factors. The conditioned media was analyzed using the Mouse Cytokine Array C3 kit (Ray Biotech) according to the manufacturer's protocol. Briefly, the cytokine array membranes were incubated in blocking buffer for 30 min at room temperature, followed by overnight incubation in 2 mL of conditioned media at 4°C. After washing, the

membranes were sequentially incubated with Biotinylated Antibody Cocktail and HRP-Streptavidin for 2 hours at room temperature, followed by chemiluminescence detection with an iBright Imaging System (Thermo Fisher Scientific). The intensity of each cytokine-specific antibody spot was measured using Fiji and the *Protein Array Analyzer* plugin. IFN-β secretion was examined using Meso Scale Discovery (MSD) U-PLEX Mouse IFN-β Assay kit (K152G0K) according to the manufacture's protocol. Briefly, 25 μL conditioned media was added to each well of MSD plate and incubated at room temperature for 1 h with shaking. After washing, 50 μL of detection Antibody Solution was added and incubated for 1 h, followed by developing with MSD GOLD Read Buffer B using an MSD instrument.

Splenocyte cells-as-sensors assay

To obtain splenocytes, spleens were isolated from BALB/c mice bearing subcutaneous CT26 tumors, dissociated, filtered through a 40 μm cell strainer, and resuspended in basic murine immune cell culture medium. The red blood cells were lysed using Red Blood Cell Lysis Buffer (Biolegend) according to the manufacturer's protocol. CT26 SnCs were treated with IR + veliparib as described above, while proliferating CT26 cells treated with DMSO were used as controls. Both SnCs and proliferating cells were washed twice with PBS, replaced with fresh murine immune cell culture medium, then cocultured with splenocytes for 3 days. Nonadherent cells were collected and diluted to 0.5 x 10^6 cells/mL. Single cell RNA sequencing was performed using 10X Genomics Chromium technology, libraries were formed and sequenced, and data were analyzed using the Seurat package in R. Genes were considered significantly upregulated or downregulated if they displayed fold change > 1.5 and P value < 0.05, comparing coculture with SnCs or proliferating cells. The list of differentially expressed genes (DEGs) was subjected to Gene Ontology (GO) analysis by g:Profiler to detect

enriched pathways.

Phagocytosis/trogocytosis assays

Senescent and proliferating cells were prepared as described above, then labeled with PKH26 (Sigma) or pHrodo Red (IncuCyte) for 15 min at 37 °C according to the manufacturers' protocols. The cells were washed and cocultured with BMDCs in a ratio of 1:2 for 6 h. Nonadherent cells were harvested, stained with fluorophore conjugated CD11c antibody alone or in combination with CD103 antibody (BioLegend) for 45 min at 4 °C. Cells were analyzed using BD Fortessa 4-15 HTS Flow cytometer and FlowJo software. The uptake of senescent cells by BMDCs was determined using a gating strategy that allows for analysis of $CD11c^+$ or $CD11c^+/CD103^+$ single cells. Detailed information on primary antibodies is provided in Supplementary Table 1.

Analysis of BMDC maturation and STING dependence

To form a STING knockout population of CT26 cells, a set of three pre-designed sgRNAs targeting mouse *Tmem173* (*Sting1*), negative control scrambled sgRNA, and spCas9 nuclease were purchased from Synthego Corporation. For transfection, sgRNAs and spCas9 were mixed in a 3:1 ratio and incubated at room temperature for 15 min to form ribonucleoprotein (RNP) complexes, which were then delivered to CT26 cells using the Neon Electroporation System (Thermofisher Scientific) according to the manufacturer's protocol. Briefly, 2×10^5 CT26 cells in 10 µL Buffer R (Thermofisher Scientific) were added to 3 µL RNPs. 10 µL of the mixture was electroporated at settings of 1600 volts, 10 ms pulse length, and 3 pulses, then immediately transferred into pre-warmed cell culture medium. Cells were allowed to recover overnight. 95% editing efficiency leading to knockout was confirmed through Sanger sequencing analysis. Loss of STING protein expression was verified by Western Blot analysis. The

resulting knockout population was then used within five passages. Detailed primer information is provided in Supplementary Table 2.

To inhibit STING, CT26 or 4T1 cells were treated with senescence inducers or DMSO controls in the presence or absence of the STING inhibitor C178 (4 μM) on Day 0, then used for BMDC coculture assay on Day 5. To activate STING, we treated CT26 cells with DMSO vehicle or senescence inducers from Day 0, then added DMXAA (30 μg/mL) on Day 3 and continued culture for another 2 days. To produce damaged cells, CT26 cells were treated with 12 Gy IR or IR + veliparib (12 Gy + 20 μM), then utilized 1 day later for BMDC coculture experiments. To prepare apoptotic senescent cells, senescent CT26 cells induced by IR + veliparib were treated with ABT-263 (5 μM) overnight.

To prepare cell lysate (CL), 2.5 x 10^6 senescent cells or proliferating controls were suspended in 1 mL PBS, followed by freezing and thawing five times and centrifugation at 12,000 g/min for 10 min at 4°C. 20 or 200 μL supernatants were used for DC coculture assays. In addition, 1 mL conditioned media (CM) prepared as in SASP characterization assays was used for DC stimulation.

All tumors cells were washed twice with PBS, then cocultured with BMDCs for 12-16 h in a 1:2 ratio in basic immune cell culture medium. After coculture, nonadherent cells were collected and incubated with zombie yellow dye (BioLegend) for 10 min at room temperature, followed by staining with CD11c, CD103, CD86, CD80, PD-L1, and H-2K^d or H-2L^d MHC I antibodies for 45 min at 4°C. Cells were analyzed using a BD Fortessa 4-15 HTS Flow cytometer and FlowJo software.

Analysis of T cell cross-priming and proliferation

To prepare CT26 immunized mice, 0.5 x 10^6 CT26 or 4T1 cells were lethally irradiated (20 Gy) and then injected subcutaneously (s.q.) into 7-9 week BALB/c mice

twice over a 10-day interval. Splenocytes were isolated from immunized mice and stained with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at room temperature. After washing, CFSE labeled splenocytes were cocultured for 5 days in a 20:1 ratio with BMDCs pre-stimulated by senescent CT26 cells or proliferating controls. After coculture, cells were collected and incubated with zombie yellow dye (BioLegend) for 10 min at room temperature, followed by staining with CD4 and CD8a antibodies for 30 min at 4°C. Cells were analyzed using a BD Fortessa 4-15 HTS flow cytometer and FlowJo software.

Time-lapse live-cell analysis

5 x 10^4 cells per well were seeded in 6-well plates, and treated with DMSO vehicle, etoposide (2 μM), GSK461364 (5 μM), IR (12 Gy), or IR + veliparib (12 Gy + 20 μM), in the absence or presence of C178 (4 μM). The plates were then analyzed by time-lapse imaging in a IncuCyte S3 live-cell imaging system (Sartorius). Phase contrast images were acquired at 10× magnification with scanning every 2 h for 3 days. More than 16 non-overlapping fields were captured for each well. Quantitative analysis of cell confluency was performed using IncuCyte S3 2020 software.

Western blotting

To verify STING knockout (KO), 2 x 10^5 STING KO cells or Scramble controls were harvested. To examine the STING/TBK1/IRF3 signaling pathway activation, 5 x 10^4 cells were seeded per well in 6-well plates, treated with DMSO vehicle, etoposide (2 μM), GSK461364 (5 μM), IR (12 Gy), or IR + veliparib (12 Gy + 20 μM) and then harvested 5 days later. To examine the effects of DMXAA, 5 x 10^4 CT26 cells were seeded per well in 6-well plates, treated with DMAXX (30 μg/mL) and harvested 2 days later. The whole-cell lysates were prepared using RIPA lysis reagent (Thermo Fisher

Scientific) in the presence of protease and phosphatase inhibitors (Thermo Fisher Scientific). 15 µg of protein was loaded per well, separated on a NuPage 4-12% Tris-Base precast gel (Invitrogen), and transferred onto a nitrocellulose membrane (Millipore). After dividing the blots into strips, immunoblotting was performed using anti-pIRF3 (phospho-Ser396), anti-IRF3, anti-γH2AX, anti-β actin, anti-STING, anti-pTBK1 (phospho-Ser172), anti-TBK1, anti-α tubulin primary antibodies as indicated, then detected with peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific, NA934vs or NA931) followed by luminescence detection using ECL substrate and an iBright Imaging System (Thermo Fisher Scientific). Detailed information on primary antibodies is provided in Supplementary Table 1.

γH2AX DNA damage foci and cytosolic DNA staining

WT or STING KO CT26 cells were seeded on sterile cover glass at $1x10^4$ per well in 24-well plates. Cells were treated for 5 days with DMSO control, veliparib alone (20 µM), IR (12 Gy), or IR + veliparib (12 Gy + 20 µM), etoposide (2 µM), or GSK461364 (5 µM), and then fixed with 4% PFA for 10 min at room temperature. For γH2AX foci staining, cells were permeabilized with 0.2% Triton-X for 10 min. After blocking with 5% BSA-PBS, cell slides were incubated overnight at 4°C with primary antibody against γH2AX (Millipore, 05-636, 1:1000) diluted in 5% BSA. For cytosolic DNA staining, 5% BSA-PBS supplemented with 0.1% Saponin was used for blocking and antibody dilution. Cell samples were blocked for 1 h at room temperature, followed by overnight incubation with primary antibody against dsDNA (Santa Crutz, HYB331-01, 1:100) at 4°C. Following PBS washes, DAPI (1 mg/mL) and fluorescent secondary antibodies (Jackson ImmunoResearch, 1:2000,) diluted in 5% BSA-PBS ± 0.1% Saponin were applied for 1 h at room temperature. Cell slides were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific) after PBS washes. Images

were captured on a Zeiss Axiovert 40CFL with a 40X Plan-NeoFluar objective and pseudo-colored using Fiji. Three replicates were performed. Detailed information on primary antibodies is provided in Supplementary Table 1.

Flow cytometric analysis of AH-1 specific T cells

7-8 week BALB/c mice received subcutaneous (s.q.) injection of 0.5 x 10^6 CT26 SnCs or PBS control. 2 days later, the draining inguinal lymph nodes were isolated, dissociated and stained with FITC AH-1 dextramer (Immudex) according to manufacturer's protocol. Briefly, ~ 2 x 10^6 cells in 50 μL staining buffer were incubated with AH-1 dextramer for 10 min at room temperature, then Alexa Fluor 647 CD8 antibody (Clone KT15, BioRad) was added for another 20 min in the dark. After washing, cells were analyzed using BD Fortessa 4-15 HTS flow cytometer and FlowJo software.

Preventative and therapeutic vaccination with SnCs or SnC-activated DCs

For preventative vaccination, 7-8 week BALB/c or NSG mice received subcutaneous (s.q.) injection of 0.5 x 10^6 CT26 SnCs in 100 μL PBS or PBS alone on Day -5, and/or intravenous (i.v.) injection of α-PD-L1 antibody (BioXCell, 0.2 mg in 100 μL PBS) on Day -4. The naive or vaccinated mice were challenged by s.q. injection of 0.5 x 10^6 CT26 or 4T1 cells in 100 μL PBS on Day 0. Tumor growth was monitored every 2-3 days for 4 weeks and the tumor volume was measured using a caliper from day 7 after tumor inoculation.

For therapeutic vaccination, 7-8 week BALB/c mice were inoculated s.q. with 0.25 or 0.5 x 10^6 CT26 or 4T1 cells in 100 μL PBS on Day 0. To initiate treatment, 0.5 x 10^6 SnCs or SnC-activated DCs in 100 μL PBS were injected peritumorally on Days 9 or 12, at tumor volumes of ~ 60 mm^3 or 150 mm^3, and then again on Days 14 or 19,

respectively. Where indicated, on Days 12 or 15, mice also received a single i.v. injection of α-PD-L1 antibody (0.2 mg) or a single 10 Gy radiation delivered using a RadSource RS-2000 X-Ray generator operating at 160 kV and 25 mA, calibrated by NIST traceable dosimetry. Tumor volume was measured using calipers every 2-3 days from day 7 after tumor inoculation.

In tumor re-challenge experiments, naive controls or mice whose tumors were eradicated by treatment with SnC-activated DC vaccine and irradiation and that remained tumor free for >10 days were injected s.q. with 0.5 x 10^6 CT26 cells on the back. Tumor growth was monitored every 2-3 days for 3 weeks.

Flow cytometric analysis of tumor infiltrating immune cells

CT26 or 4T1 tumors were collected 5 days after their last treatment and divided in half for either flow cytometric or histological analyses. For flow cytometry, the tumors were dissociated using a Miltenyi Tumor Dissociation Kit. Briefly, tumor tissues were transferred into the gentleMACS C Tubes containing enzyme mix. Then the C tubes were run on a gentleMACS Dissociator using gentleMACS program m_impTumor_02. After termination of the program, samples were incubated for 30 min at 37 °C. After dissociation, the cell suspensions were filtered through a 70 μm cell strainer and pelleted by centrifugation at 300 × g for 5 min. The cell pellet was resuspended in PBS, incubated with zombie yellow dye (BioLegend) for 10 min at room temperature, washed, and stained with fluorophore conjugated CD45, CD3, CD4, CD8, CD49b, CD11c, and CD103 antibodies for 30 min at 4°C, followed by analysis using a BD Fortessa 4-15 HTS flow cytometer and FlowJo software. Detailed information on primary antibodies is provided in Supplementary Table 1.

Lung colonization assays

7-8 week female BALB/c mice were inoculated intravenously (i.v.) with 6 x 10^4 or 8 x 10^4 proliferating 4T1 cells on Day 0, delivering disseminated tumor cells to the lungs. For preventative vaccination, 0.5 x 10^6 4T1 SnCs in 100 µL PBS were injected subcutaneously (s.q.) on Days -5 and -1. For therapeutic vaccination, 0.5 x 10^6 4T1 SnCs or 0.5 x 10^6 4T1 SnC-activated DCs in 100 µL PBS were injected s.q. on Days 1 and 5. Where indicated, mice also received a single i.v. dose of α-PD-L1 antibody (0.2 mg) on Day 3. On Day 21, mice were euthanized, lungs were perfused with 1 mM EDTA-PBS, fixed with 10% neutral formalin, and then examined visually for surface metastatic foci and/or embedded and sectioned for histological analysis to evaluate colonization in lung parenchyma.

Histology and immunofluorescence

The remaining halves of the CT26 or 4T1 tumors were fixed with 10% neutral formalin for histological analysis. Alternatively, mouse lungs were collected, perfused with 1 mM EDTA-PBS and fixed with 10% neutral formalin. Tissue processing, embedding, and sectioning were performed by the Human Tissue Resource Center at the University of Chicago. 5 µm sections were stained with hematoxylin and eosin (H&E) and scanned using a CRi Pannoramic SCAN 40x Whole Slide Scanner. For immunofluorescence, tumor sections were deparaffinized with xylene, rehydrated, and immersed in 10 mM sodium citrate buffer (pH 6.0) for 30 min at 90 °C for antigen retrieval. After blocking with 5% BSA, the samples were stained with CD8, perforin, and granzyme B primary antibodies at 4 °C overnight, washed, then stained with fluorophore-conjugated secondary antibodies (Vector Labs), counterstained with DAPI, mounted and scanned using an Olympus VS200 SlideView Whole Slide Scanner.

Supplementary figure legends

Supplementary Figure 1

SnCs primarily affect conventional type 1 dendritic cells

A, SA-β-Gal staining of CT26 cells. CT26 cells were treated with DMSO vehicle or IR + veliparib (12 Gy + 20 μM), followed by fixation and staining 5 days later. The mean ± SD percentage of SA-β-Gal-positive SnCs in five 20× fields is indicated. Scale bars: 200 μm. **B**, Experimental schema for scRNA-seq analysis of splenocytes after coculturing with CT26 proliferating controls or SnCs prepared as in **A** with 12 Gy + veliparib. **C,** tSNE analysis of scRNA-seq of nonadherent cells after splenocytes were cocultured with proliferating controls (cyan, n=593) or SnCs (red, n=1024) for 3 days. **D**, Stacked bar graph showing the relative proportion of 11 cell clusters after coculturing with proliferating cells or SnCs. cDC2, conventional type 2 dendritic cell. HSC, hematopoietic stem cell. APC, antigen-presenting cell. **E**, Scatter plots from scRNA-seq clusters showing the differential gene expression (DGE) in splenocytes cocultured with proliferating controls or SnCs. Dots indicate relative expression in each cluster for a single detected gene. Blue lines indicate equal expression and R^2, the goodness of fit. All genes that passed quality control were analyzed. cDC1, conventional type 1 dendritic cell. cDC2, conventional type 2 dendritic cell. **F**, Reactome Gene Ontology (GO) analysis and **G**, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of upregulated differentially expressed genes (DEGs) in cDC1 after coculturing with SnCs confirm Biological Process-enriched pathways. Dots indicate the number of DEGs and bars the -Log10 (p-value) for each enriched pathway.

Supplementary Figure 2

Senescent cells secrete more cytokines compared to proliferating cells

A, Cytokine array analysis of the conditioned media. CT26 cells were treated for 4 days with DMSO vehicle, etoposide (2 μM), GSK461364 (5 μM), IR alone (12 Gy), or IR + veliparib (12 Gy + 20 μM), washed, then continuously cultured in fresh medium for 2 days to collect the conditioned media. Color-coded rectangle frames indicate the position of SASP factors that have dramatically changed in senescent cells compared to proliferating cells. **B**, Complete list of cytokines detected by the array.

Supplementary Figure 3

Gating strategy of flow cytometric analysis

A, To examine the effects of coculture, cells were first gated based on forward scatter (FSC-A and FSC-H) for the single cell population. Then $CD11c^+$ DCs were selected for quantification analysis of PKH26 intensity. **B**, Cells were first gated for single live cells by size and Zombie yellow exclusion, then gated on co-expression of CD11c and CD103 to identify DCs. The DC population was analyzed for pHrodo Red intensity. **C**, DCs were identified as in **B**, and then analyzed for surface expression of activation/maturation markers CD80, CD86, H-$2K^d$, and PD-L1. **D**, To evaluate T cell proliferation, cells were gated on size and viability and then further gated into $CD8^+$ and $CD4^+$ T cell populations. CFSE dilution was measured in both $CD8^+$ and $CD4^+$ T cells. **E**, To examine the effects of ABT263, CT26 SnC single-cell population was selected based on forward scatter (FSC-A and FSC-H), and apoptosis was analyzed by Annexin V binding and propidium iodide (PI) uptake. **F**, To examine CT26-reactive CTLs, cells gated on size and $CD8^+$ expression were analyzed for AH-1 dextramer binding. **G**, Dissociated tumor cells gated on size, viability, and CD45 were evaluated for NK cells ($CD49b^+$/$CD3^-$) and T cells ($CD3^+$/$CD49b^-$), which were further gated into $CD8^+$ and

$CD4^+$ T cell populations. **H**, Live $CD45^+$ immune cells were gated as in **G,** followed by the identification of dendritic cells (DCs, $CD11c^+/CD103^+$).

Supplementary Figure 4

SnCs are effectively engulfed by BMDCs *in vitro*

To evaluate uptake of SnCs by BMDCs, CT26 (**A**) or 4T1 (**B**) SnCs were induced by IR (12 Gy), IR + veliparib (12 Gy + 20 μM), or etoposide (2 μM). Proliferating CT26 and 4T1 cells were used as controls. BMDCs were cocultured with pHrodo Red-labeled cells for 6 h, followed by staining and flow cytometric analysis. The geometric mean fluorescence intensity (MFI) of pHrodo Red was determined in the single, viable, $CD11c^+/CD103^+$ cell population. Data from three experiments, mean ± SD. *** $P < 0.001$, ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$ (paired t-test).

Supplementary Figure 5

Senescent 4T1 cells promote DC maturation/activation and T cell priming *in vitro*

A, SA-β-Gal staining of 4T1 cells. 4T1 cells treated with DMSO vehicle or veliparib (20 μM) were used as controls. Cellular senescence was induced by IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or GSK461364 (5 μM). The mean ± SD percentage of SA-β-Gal-positive cells from five 20× fields is indicated. Scale bars: 200 μm. **B**, Phagocytosis assays indicating effective uptake of senescent cells (SnCs) by BMDCs. 4T1 SnCs and controls were prepared as in **A**. BMDCs were cocultured with PHK26 labeled 4T1 cells for 6 h, followed by staining and flow cytometric analysis to determine PHK26 MFI in the $CD11c^+$ DC population. Data from three experiments, mean ± SD. **C**, For quantitative analysis of DC activation/maturation, BMDCs were cocultured overnight with 4T1 SnCs and controls as prepared in **A**, stained for CD80, CD86, $H\text{-}2K^d$, and PD-L1, and analyzed by flow cytometry to determine MFI in single,

viable, CD11c$^+$/CD103$^+$ DCs. Data from three experiments, mean ± SD. For statistical analysis, *** $P < 0.001$, ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$ (paired t-test). **D** and **E**, T cell priming by SnC-activated DCs. CFSE-labeled splenocytes were cocultured for 5 days with DCs stimulated by 4T1 SnCs or controls as in **C,** in the absence or presence of α-PD-L1. Shown are the % proliferative (CFSE diluted) fraction of viable CD8$^+$/CD4$^-$ (**D**) or CD8$^-$/CD4$^+$ (**E**) T cells.

Supplementary Figure 6

T cell priming by CT26 SnC-loaded DCs

Primary flow cytometry data linked to **Fig. 2F** and **G**. CFSE-labeled splenocytes cocultured for 5 days with DCs pre-stimulated by CT26 proliferating or senescent cells in the absence (**A** and **C**) or presence (**B** and **D**) of α-PD-L1. Shown are zombie yellow$^-$/CD8$^+$/CD4$^-$ (**A** and **B**) and zombie yellow$^-$/CD4$^+$/CD8$^-$ (**C** and **D)** T cell populations, indicating % proliferating cells.

Supplementary Figure 7

T cell priming by 4T1 SnC-loaded DCs

Primary flow cytometry data linked to **Fig. S3E** and **F.** CFSE-labeled splenocytes cocultured for 5 days with DCs pre-stimulated by proliferating or senescent 4T1 cells in the absence (**A** and **C**) or presence (**B** and **D**) of α-PD-L1. Shown are zombie yellow$^-$/CD8$^+$/CD4$^-$ (**A** and **B**) and zombie yellow$^-$/CD4$^+$/CD8$^-$ (**C** and **D)** T cell populations, indicating % proliferating cells.

Supplementary Figure 8

DC stimulation by SnC lysates and conditioned media and increased cytoplasmic DNA, STING activation, and IFN-β secretion in SnCs

A, BMDCs were cocultured overnight with cells, cell lysates (CL) or conditioned media (CM) derived from proliferating controls or senescent cells. Surface expression of CD80, CD86, H-2K^d, and PD-L1 was analyzed by flow cytometry to determine MFI in single viable CD11c$^+$/CD103$^+$ DCs. Data from three experiments, mean ± SD. **B** and **C,** Representative pseudo-colored images of staining for DNA damage marker γH2AX (red) (**B**) or cytoplasmic DNA (red) (**C**), overlaid with DAPI (blue). Cell contours on bright-field images are indicated by yellow dotted lines (**C**). CT26 cells were treated for 5 days with DMSO vehicle, veliparib alone (20 μM), IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or GSK461364 (5 μM). Scale bars: 20 μm. **D**, Western blot analysis indicates the upregulation of γH2AX and IRF3 phosphorylation in senescent cells. Cells were treated as in **B**. Shown are representative Western blot results for γH2AX (phospho-Ser139), p-IRF3 (phospho-Ser396), total IRF-3, and β-tubulin loading control of whole-cell lysates, loaded with 15 μg protein per lane. **E**, Meso Scale Discovery (MSD) Immunoassay analysis of IFN-β secretion in the conditioned media. CT26 cells were treated as in **B** for 4 days, then washed and cultured in fresh medium for another 2 days to collect the conditioned media. Data from four experiments, mean ± SD. For statistical analysis, *** $P < 0.001$, ** $0.001 < P < 0.01$ (paired t-test).

Supplementary Figure 9

STING is not required for senescence induction in CT26 cells

A, Western blot verification of STING knockout (KO) in CT26 cells. CT26 cells were electroporated with RNPs formed with gRNAs targeting *Teme173* (*Sting1*) or scramble gRNA control. Shown are representative Western blot results of whole-cell lysates from passage 5 after transfection. **B** and **C**, Automated cell growth analysis from time-lapse imaging over 3 days. Scramble (Scr, **B**) and STING KO CT26 cells (**C**) were treated with DMSO, IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or

GSK461364 (5 μM) at time 0. Results are shown as mean ± SEM. Images of 16 non-overlapping fields were captured for analysis of each sample. **D**, SA-β-Gal staining of CT26 cells. Scr or STING KO CT26 cells were treated as in **B** and **C**, followed by fixation and staining after 5 days. The mean ± SD percentage of SA-β-Gal-positive cells from five 20× fields is indicated. Scale bars: 200 μm. **E** and **F**, Representative pseudo-colored images of staining for DNA damage marker γH2AX (red) (**E**) or cytoplasmic DNA (red) (**F**), overlaid with DAPI (blue). Cell contours on bright-field images are indicated by yellow dotted lines (**F**). Scr or STING KO CT26 cells were treated as in **D**. Scale bars: 20 μm.

Supplementary Figure 10

STING signals modulate the expression of CCL5 and MHC class I molecules in senescent CT26 cells

A, Cytokine array analysis of the conditioned media. STING KO CT26 cells were treated with DMSO vehicle, etoposide (2 μM), or IR + veliparib (12 Gy + 20 μM) for 4 days, then washed and cultured in fresh medium for another 2 days to collect the conditioned media. Color-coded rectangle frames indicate the position of SASP factors that have dramatically changed in senescent cells compared to proliferating cells. **B**, Quantitative analysis of cytokine secretion by non-senescent or senescent Scramble or STING KO CT26 cells. **C**, Quantitative analysis of MHC I molecules expression. Scramble or STING KO CT26 cells were treated for 5 days with DMSO, IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or GSK461364 (5 μM), then stained for H-2K^d, H-2D^d, or H-2L^d and analyzed by flow cytometry to determine MFI in single cell population. Data from three experiments, mean ± SD. *** $P < 0.001$, ** $0.001 < P < 0.01$, n.s. $P > 0.05$ (paired t-test).

Supplementary Figure 11

STING is required for CT26 SnCs to activate DCs

A, Automated cell growth analysis from time-lapse imaging. CT26 cells were treated with DMSO, IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or GSK461364 (5 μM) in the absence or presence of C178 (4 μM) at time 0. Images were captured over 3 days for each condition at a 2-hour interval. Results are shown as a fit to the mean ± SEM of 16 non-overlapping fields for each sample at each time point. **B,** SA-β-Gal staining of CT26 cells. Cells were treated as in **A**, followed by fixation and staining after 5 days. The mean ± SD percentage of SA-β-Gal-positive cells from five 20× fields is indicated. Scale bars: 200 μm. **C**, Quantitative analysis of DC activation/maturation. BMDCs were cocultured overnight with CT26 cells treated as in **A.** The single viable $CD11c^+/CD103^+$ DC population was analyzed for MFI of CD80, CD86, $H\text{-}2K^d$, and PD-L1. Data from three experiments, mean ± SD. *** $P < 0.001$, ** $0.001 < P < 0.01$, n.s. $P > 0.05$ (paired t-test).

Supplementary Figure 12

STING is required for 4T1 SnCs to activate DCs

A, Automated time-lapse imaging analysis of growth kinetics of 4T1 cells treated at time 0 with DMSO, IR (12 Gy), IR + veliparib (12 Gy + 20 μM), etoposide (2 μM), or GSK461364 (5 μM) in the absence (left) or presence (right) of C178 (4 μM). Shown is the non-linear fit to the mean ± SEM of 16 non-overlapping fields for each sample at indicated time point. **B**, SA-β-Gal staining of 4T1 cells. Cells were treated as in **A**, followed by fixation and staining after 5 days. The mean ± SD percentage of SA-β-Gal-positive cells from five 20× fields is indicated. Scale bars: 200 μm. **C**, Quantitative analysis of DC activation/maturation. BMDCs were cocultured overnight with 4T1 cells treated as in **A.** The single viable $CD11c^+/CD103^+$ DC population was analyzed for MFI

of CD80, CD86, H-2K^d, and PD-L1. Data from three experiments, mean ± SD. *** P < 0.001, ** 0.001 < P < 0.01, n.s. P > 0.05 (paired t-test).

Supplementary Figure 13

STING/TBK1/IRF3 signaling activation in tumor cells is not sufficient for DC activation but is necessary for senescent cells to serve as protective vaccines

A, The STING agonist DMXAA (30 µg/mL) activates STING signals in CT26 cells after two days of treatment. Shown are representative Western blot results for p-TBK1 (phospho-Ser172), total TBK1, and β-actin loading control of whole-cell lysates, loaded with 15 µg protein per lane. **B**, Quantitative analysis of DC activation/maturation. Cellular senescence was induced using etoposide (2 µM) or IR + veliparib (12 Gy + 20 µM). The non-senescent or senescent Scramble or STING KO CT26 cells were pretreated for 2 days with or without DMXAA (30 µg/mL), washed, and then cocultured overnight with BMDCs. The Zombie yellow-/CD11c+/CD103+ DC population was analyzed for MFI of CD80, CD86, H-2K^d, and PD-L1. Data from three experiments, mean ± SD. *** P < 0.001, ** 0.001 < P < 0.01, * 0.01 < P < 0.05, n.s. P > 0.05 (paired t-test). **C**, BALB/c mice injected with 0.5 x 10^6 Scramble (Scr) or STING KO CT26 SnCs at Day -5 were challenged with 0.5 x 10^6 CT26 proliferating cells on Day 0 and examined for palpable tumors at 2-3 day intervals (n = 7 per group). Shown is Kaplan-Meier analysis of CT26 tumor incidence over time. 6/7 Scr SnC-vaccinated mice and 2/7 STING KO-vaccinated mice did not develop tumors. Logrank test. *** P < 0.001, * 0.01 < P < 0.05.

Supplementary Figure 14

CT26 SnC vaccines suppress tumor growth and potentiate cancer therapies

Individual tumor growth data linked to **Fig. 5A-F. A,** Experimental schema for treating CT26 tumor bearing mice with SnC vaccine and/or α-PD-L1, initiating treatment at Day 9. **B-E**, Growth kinetics of individual CT26 tumors untreated (**B**), treated with SnC vaccine (**C**), α-PD-L1 (**D**), or the combination therapy (**E**). **F**, Experimental schema for treating CT26 tumor bearing mice with SnC vaccine and/or 10 Gy irradiation (IR). **G-J**, Growth kinetics of individual CT26 tumors untreated (**G**), treated with SnC vaccine (**H**), IR (**I**), or the combination therapy (**J**).

Supplementary Figure 15

CT26 SnC vaccines suppress tumor growth and potentiate cancer therapies

A, Experimental schema for treating CT26 tumor bearing mice with SnC vaccine and/or α-PD-L1, initiating treatment at Day 12. **B,** Growth kinetics of CT26 tumors untreated, treated with SnC vaccine, α-PD-L1, or combination therapy (n = 5-7 per group, mean ± SEM). **C**, CT26 tumor weight at Day 30. Shown are individual tumor weights (circle) and weight range (box and whisker). **D-G**, Individual tumor growth data linked to **B.** Shown are growth kinetics of individual CT26 tumors untreated (**D**), treated with SnC vaccine (**E**), α-PD-L1 (**F**), or the combination therapy (**G**). **H**, Experimental schema for treating CT26 tumor bearing mice with SnC vaccine and/or 10 Gy irradiation (IR), initiating treatment at Day 12. **I**, Growth kinetics of CT26 tumors untreated, treated with SnC vaccine, IR, or combination therapy (n = 5-7 per group, mean ± SEM). **J**, CT26 tumor weight at Day 30. Shown are individual tumor weights (circle) and weight range (box and whisker). **K-N**, Individual tumor growth data linked to **I.** Shown are growth kinetics of individual CT26 tumors untreated (**K**), treated with SnC vaccine (**L**), IR (**M**), or combination therapy (**N**). For statistical analysis, *** $P < 0.001$, ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$, n.s. $P > 0.05$ (paired t-test).

Supplementary Figure 16

Senescent 4T1 cell vaccines suppress tumor growth and potentiate immunotherapy

A, Experimental schema for treating 4T1 tumor-bearing mice with SnC vaccine and/or α-PD-L1. **B,** Growth kinetics of 4T1 tumors untreated, treated with SnC vaccine, α-PD-L1, or the combination therapy (n = 5-7 per group, mean ± SEM). **C**, 4T1 tumor size at Day 30. Shown are individual tumor sizes (circle) and size range (box and whisker). *** P < 0.001, ** 0.001 < P < 0.01, * 0.01 < P < 0.05, n.s. P > 0.05 (paired t-test). **D-G**, Individual tumor growth data linked to **B.** Growth kinetics of individual 4T1 tumors untreated (**D**), treated with SnC vaccine (**E**), α-PD-L1 (**F**), or combination therapy (**G**).

Supplementary Figure 17

CT26 SnC vaccine promotes CTL infiltration and activation

A, Representative H&E staining of CT26 tumor sections. CT26 tumors excised at Day 18 (5 days after second SnC injection) were divided, fixed, embedded and sectioned. Serial sections as in **Fig. 5H** were used. Shown are representative whole section scanning (upper panel, scale bar: 2 mm) and selected enlarged regions (lower panel, scale bar: 200 μm). **B**, Immunofluorescence staining of serial sections to H&E in **A** for activated CTL infiltrate with markers CD8 (red), granzyme B (green), and DAPI (blue). Scale bars: 200 μm.

Supplementary Figure 18

4T1 SnC vaccine promotes CTL infiltration and activation

A, Analysis of immune infiltrate in 4T1 tumors. Mice bearing subcutaneous 4T1 tumors were untreated or treated with SnC vaccine, α-PD-L1, or combination therapy as in **Fig. S2**. Tumors excised at Day 18 (5 days after second SnC injection) were divided,

dissociated and stained for flow cytometry. The viable, single cell population was analyzed for total immune ($CD45^+$), DC ($CD11c^+/CD103^+$), T_h ($CD3^+/CD4^+/CD8^-$), CTL ($CD3^+/CD8^+/CD4^-$), and NK ($CD3^-/CD49b^+$) cells. Shown are individual tumors (open circles, n = 3 per group) and mean ± SD (bar). *** $P < 0.001$, ** $0.001 < P < 0.01$, * $P < 0.05$, n.s. $P > 0.05$ (paired t-test). **B**, The remaining portion of the 4T1 tumors examined in **A** were fixed, embedded and sectioned. Shown are representative H&E whole section scanning (scale bar: 2 mm) and selected enlarged regions (scale bar: 200 µm). **C** and **D**, Immunofluorescence staining of serial sections to the H&E in **B** for activated CTL infiltrate with (**C**) markers CD8 (yellow), perforin (red), and DAPI (blue), or (**D**) CD8 (red), granzyme B (green), and DAPI (blue). Scale bars: 200 µm.

Supplementary Figure 19

4T1 SnC vaccines suppress lung colonization

A, Experimental schema for treating with SnC vaccines to limit lung colonization. BALB/c mice were injected i.v. with 8×10^4 4T1 proliferating cells on Day 0 and then treated with 0.5×10^6 4T1 SnCs on Days -5 and -1 or Days 1 and 5 as preventative or therapeutic vaccines, respectively. Lungs were collected on Day 21. **B**, Quantification of metastatic foci on lung surface. Shown are counts from individual lungs (dot), with mean ± SEM (bar). ** $0.001 < P < 0.01$, * $P < 0.05$ (paired t-test). **C**, Representative H&E staining of colonization at low magnification with inset zoomed to high magnification. Scale bars: 2 mm (upper) and 50 µm (lower).

Supplementary Figure 20

SnC-activated DC vaccine potentiates radiotherapy

Individual tumor growth data linked to **Fig. 7A-C. A,** Experimental schema for treating CT26 tumor bearing mice with SnC-activated DC vaccine and/or IR. **B-E**, Growth

kinetics of individual CT26 tumors untreated (**B**), treated with SnC-activated DC vaccine (**C**), IR (**D**), or combination therapy (**E**).

Supplementary Table 1
List of antibodies used

Antibody	**Company**	**Catalog #**	**Dilution**
Flow Cytometry			
Pacific Blue™ anti-mouse CD11c	BioLegend	117321	1:100
Brilliant Violet 711 anti-mouse CD103	BioLegend	121435	1:100
PE/Cyanine7 anti-mouse CD86	BioLegend	105013	1:100
APC anti-mouse CD8a	BioLegend	100711	1:100
CD274 (PD-L1, B7-H1) antibody	Invitrogen	12-5982-81	1:100
Alexa Fluor 488 anti-mouse H-2K^d	BioLegend	116609	1:100
PE anti-mouse H-2L^d/H-2D^b	BioLegend	114507	1:100
PE anti-mouse H-2D^d Antibody	BioLegend	110607	1:100
Alexa Fluor 700 anti-mouse CD45	BD Bioscience	560510	1:100
Pacific Blue™ anti-mouse CD3	BioLegend	100213	1:100
APC anti-mouse CD49b	BioLegend	108909	1:100
PE/Cyanine7 anti-mouse CD4	BioLegend	100421	1:100
BB515 anti-mouse CD8a	BD Bioscience	564422	1:100
Alexa Fluor 647 anti-mouse CD8α	BioRad	MCA609	1:100
Immunofluorescence			
CD8	Invitrogen	MA1-10301	1:500
Perforin	Cell Signaling Technology	44865	1:500
Granzyme B	Cell Signaling Technology	44153	1:500
Anti-phospho-Histone H2A.X (Ser139) Antibody, clone JBW301	Millipore Sigma	05-636	1:1000
ds DNA Marker Antibody (HYB331-01)	Santa Cruz	sc-58749	1:200
Western Blot			
Phospho-IRF3 (Ser379)	Cell Signaling Technology	4947	1:1000
IRF-3 (D83B9)	Cell Signaling Technology	4302	1:1000
Phospho-Histone H2A.X (Ser139)	Cell Signaling Technology	9718	1:1000
STING	Cell Signaling Technology	13647	1:1000
Phospho-TBK1 (Ser172)	Cell Signaling Technology	5483	1:1000
TBK1/NAK (E8I3G)	Cell Signaling Technology	38066	1:1000
β-actin (HRP conjugate)	Proteintech	HRP-60008	1:5000
α-tubulin (HRP conjugate)	Proteintech	HRP-60031	1:5000

Supplementary Table 2
Sequences of oligonucleotides used

sgRNA targeting *Tmem173*	GCGAGGCUAGGUGAAGUGCU
sgRNA targeting *Tmem173*	GAUGAUCCUUUGGGUGGCAA
sgRNA targeting *Tmem173*	ACCUGCAUCCAGCCAUCCCA
Forward primer for PCR	AGGGAAGGCCAAGGTTAGGA
Reverse primer for PCR	GGCGTCTCCTTGAGGTGTAT
Sequence primer for Sanger Sequencing	GTCTCCTTGAGGTGTATCCAAGAGTAGC