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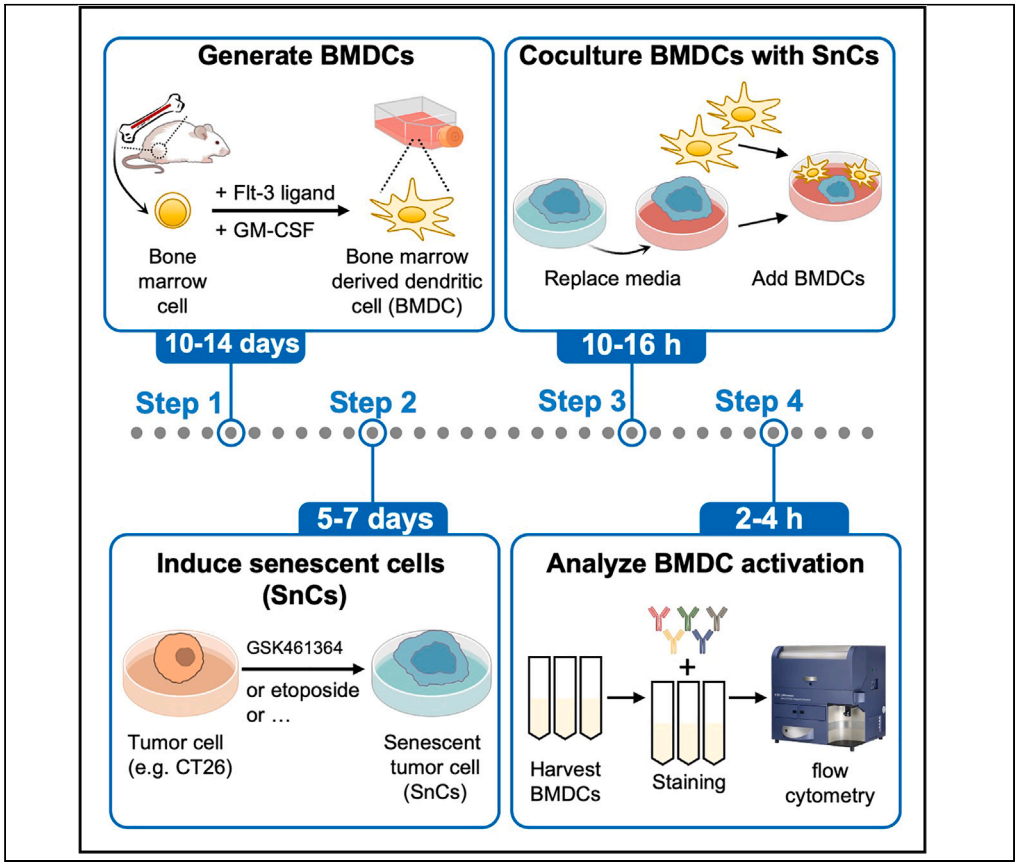
Protocol for examining the capability of senescent tumor cells to stimulate murine bone-marrow-derived dendritic cells by flow cytometry



Therapy-induced senescence (TIS) may contribute to therapy resistance; however, evidence also suggests that senescent cells (SnCs) may promote anti-tumor immunity. Here, we present a protocol for examining the capability of TIS to stimulate type 1 conventional CD103$^+$ dendritic cells (DCs). We describe steps for isolating and differentiating CD103$^+$ DCs from murine bone marrow, inducing senescence in murine colon carcinoma cell line CT26, and coculturing DCs with SnCs. We then detail the flow cytometric analysis of DC maturation and activation.

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Highlights

Treating murine tumor cells to form therapy-induced senescent cells (SnCs)

Differentiation of murine bone marrow to CD103$^+$ dendritic cells (DCs)

Coculture to evaluate SnC immunogenicity via DC maturation and activation

Analysis of BMDC activation by flow cytometry

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Protocol for examining the capability of senescent tumor cells to stimulate murine bone-marrow-derived dendritic cells by flow cytometry

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SUMMARY

Therapy-induced senescence (TIS) may contribute to therapy resistance; however, evidence also suggests that senescent cells (SnCs) may promote anti-tumor immunity. Here, we present a protocol for examining the capability of TIS to stimulate type 1 conventional CD103$^+$ dendritic cells (DCs). We describe steps for isolating and differentiating CD103$^+$ DCs from murine bone marrow, inducing senescence in murine colon carcinoma cell line CT26, and coculturing DCs with SnCs. We then detail the flow cytometric analysis of DC maturation and activation.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2022)[1] and Liu et al. (2023).[2]

BEFORE YOU BEGIN

Senescence has recently been recognized as a hallmark of cancer due to the growing literatures revealing the impact of cellular senescence on the tumor microenvironment.[3] Cells can undergo stress-induced senescence in response to genotoxic or other anti-cancer therapies, commonly referred to as therapy-induced senescence (TIS).[4] Despite their inability to proliferate, senescent cells (SnCs) remain metabolically active, display altered gene expression and surface protein presentation, and secrete a range of proteins collectively known as the senescence-associated secretory phenotype (SASP).[5] Thereby, SnCs play critical roles in modulating the anti-tumor immune responses through autocrine and paracrine mechanisms.[6] However, the existing literature presents conflicting results regarding the beneficial and detrimental influence of senescent tumor cells on anti-tumor immunity.[7] Despite not being fully understood, the immunogenic potential of senescence has been at least partially attributed to its role in activating dendritic cells (DCs).[8,9]

Dendritic cells (DCs) serve as antigen-presenting cells, connecting innate and adaptive immune responses.[10] Type 1 conventional DCs (cDC1) represent a specific subset of DCs that specialize in processing and presenting intracellular antigens via the major histocompatibility complex class I (MHC I) signaling pathway to CD8$^+$ T cells, thereby stimulating anti-tumor immunity.[11] Specifically, a CD103$^+$ cDC1 population has been found to migrate from peripheral tissues or tumors to lymph nodes where they prime naive CD8$^+$ T lymphocytes and facilitate their activity.[12–14] *In vitro* studies also demonstrate that CD103$^+$ cDC1s are highly effective at stimulating CD8$^+$ T cells against tumor-associated antigens.[13,14]

In this study, we present a protocol for evaluating the effects of senescent tumor cells on DC activation and maturation, which may serve as a facile *in vitro* proxy to predict the immunogenicity of senescent tumor cells *in vivo*. This protocol describes the steps for the *in vitro* differentiation of CD11c$^+$/CD103$^+$ DCs

from fresh isolated bone marrow of BALB/c mice, the induction of cellular senescence with BALB/c mouse-derived colorectal carcinoma CT26 cells, along with the flow cytometric analysis of DC activation induced by senescent CT26 cells. This protocol has been applied successfully with other BALB/c mouse-derived cell lines, including mammary carcinoma 4T1 cells. SnC-pulsed DCs produced through this protocol can be used for downstream applications, such as T cell priming assays or dendritic cell vaccines. This approach can be applied to other mouse strains, including C57BL/6J. Notably, it is critical to match the haplotype of the bone marrow mouse donor with cell lines used for senescence induction, especially when SnC-pulsed DCs are used in functional experiments. Time estimates listed here may vary depending on cell lines and experimentation skills.

Media and solutions should be prepared according to the materials and equipment section prior to experiments. Storage instructions are provided in the footnotes of the recipes.

Institutional permissions

All mice used for these studies were maintained and procured following guidelines approved by the University of Chicago Institutional Animal Care and Use Committee, protocol number 72354. Users of this protocol should note that permissions for experiments on live vertebrates must be obtained in advance from relevant institutional and national guidelines and regulations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pacific Blue anti-mouse CD11c antibody, 1:200	BioLegend	Cat# 117321, RRID: AB_755987
Brilliant Violet 711 anti-mouse CD103 antibody, 1:400	BioLegend	Cat# 121435, RRID: AB_2686970
APC/Cyanine7 anti-mouse I-A/I-E antibody, 1:200	BioLegend	Cat# 107627, RRID: AB_1659252
APC anti-mouse CD80 antibody, 1:200	BioLegend	Cat# 104713, RRID: AB_313134
PE/Cyanine7 anti-mouse CD86 antibody, 1:200	BioLegend	Cat# 105013, RRID: AB_439782
Alexa Fluor 488 anti-mouse H-2K^{d} antibody, 1:200	BioLegend	Cat# 116609, RRID: AB_493066
CD274 (PD-L1, B7-H1) monoclonal antibody (MIH1), PE, eBioscience, 1:100	Invitrogen	Cat# 12-5983-42, RRID: AB_11042286
TruStain FcX (anti-mouse CD16/32) antibody, 0.5 μg/100 μL	BioLegend	Cat# 101319, RRID: AB_1574973
Chemicals, peptides, and recombinant proteins		
RPMI 1640 medium	Thermo Fisher Scientific	Cat# 11875093
Fetal bovine serum (FBS)	Thomas Scientific	Cat# C788U20
Heat-inactivated fetal bovine serum	Thermo Fisher Scientific	Cat# A3840001
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# 15070063
2-Mercaptoethanol (50 mM)	Thermo Fisher Scientific	Cat# 31350010
Trypsin-EDTA	Thermo Fisher Scientific	Cat# 25300054
Dulbecco's phosphate-buffered saline (DPBS)	Corning	Cat# 21-031-CV
Distilled water	Thermo Fisher Scientific	Cat# 15230170
Recombinant murine GM-CSF	PeproTech	Cat# 315-03
Recombinant murine Flt3-ligand	PeproTech	Cat# 250-31L
Bovine serum albumin (BSA)	Sigma	Cat# A9418
70% Ethanol	Lab Alley	Cat# EAD140
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D2650
Etoposide	Selleck Chemicals	Cat# S1225
GSK461364	Selleck Chemicals	Cat# S2193
10× RBC lysis buffer	BioLegend	Cat# 420301
Ammonium-chloride-potassium (ACK) lysing buffer	Thermo Fisher Scientific	Cat# A1049201
16% Paraformaldehyde aqueous solution	Electron Microscopy Sciences	Cat# 15710
X-Gal	Gold Biotechnology	Cat# X4281C
Dimethylformamide (DMF)	Sigma	Cat# 319937

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Potassium ferricyanide ($K_3Fe(CN)_6$)	Sigma	Cat# 702587
Potassium ferrocyanide trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$)	Sigma	Cat# P3289
Citric acid	Sigma	Cat# 251275
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)	Sigma	Cat# S9390
Sodium chloride (NaCl)	Sigma	Cat# S9888
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Sigma	Cat# M2670
UltraComp eBeads Compensation Beads	Thermo Fisher Scientific	Cat# 01-2222-42
BD Horizon Brilliant Stain Buffer	BD Biosciences	Cat# 563794
Zombie Yellow Fixable Viability Kit, 1:2,000	BioLegend	Cat# 423103
Cell staining buffer	BioLegend	Cat# 420201
Experimental models: Cell lines		
Mouse: CT26.WT	ATCC	Cat# CRL-2638
Experimental models: Organisms/strains		
Mouse: 7–9 weeks female BALB/cAnNHsd	Envigo	Cat# 047
Mouse: CT26 cells	ATCC	Cat# CRL-2638
Software and algorithms		
FlowJo	BD Biosciences	https://www.flowjo.com/
Fiji	Schindelin et al. (2012)[15]	https://imagej.github.io/software/fiji/#publication
Prism	GraphPad	https://www.graphpad.com/
Other		
Falcon 40 μm cell strainer	Corning	Cat# 352340
Falcon 50 mL centrifuge tube	Corning	Cat# 352098
Falcon 5 mL round-bottom polystyrene test tube	Corning	Cat# 352054
Fisherbrand sterile syringe filters	Fisherbrand	Cat# 09-720-3
Fisherbrand 5 mL sterile syringes	Fisherbrand	Cat# 14-955-458

MATERIALS AND EQUIPMENT

Complete RPMI

Reagent	Final concentration	Amount
RPMI 1640 Medium	N/A	445 mL
Fetal bovine serum (FBS)	10%	50 mL
Penicillin-Streptomycin	1%	5 mL
Total	N/A	500 mL

Store at 4°C and use for the next 2–3 months.

Basic immune cell medium

Reagent	Final concentration	Amount
RPMI 1640 Medium	N/A	444.5 mL
Heat inactivated FBS	10%	50 mL
Penicillin-Streptomycin	1%	5 mL
2-Mercaptoethanol (50 mM)	50 μM	0.5 mL
Total	N/A	500 mL

Store at 4°C and use for the next 2–3 months.

DC medium

Reagent	Final concentration	Amount
Basic immune cell medium	N/A	20 mL
Recombinant murine Flt3 ligand (100 μg/mL)	200 ng/mL	40 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Recombinant murine GM-CSF (5 μg/mL)	1 ng/mL	4 μL
Total	N/A	20 mL
Prepare fresh before using.		

- 10% FBS-PBS: Add 100 μL heat inactivated FBS in 900 μL DPBS.

  Alternatives: 0.1% bovine serum albumin (BSA) in DPBS.

Store at −20°C for up to 6 months.

- 100 μg/mL recombinant murine Flt3 ligand solution: Reconstitute 10 μg lyophilized recombinant murine Flt3 ligand (PeproTech) in 100 μL 10% FBS-PBS.

Distribute into 20 μL aliquots and store at −80°C for up to 3 months.

- 10 μg/mL recombinant murine GM-CSF solution: Reconstitute 5 μg lyophilized recombinant murine GM-CSF (PeproTech) in 500 μL 10% FBS-PBS.

Distribute into 10 μL aliquots and store at −80°C for up to 3 months.

⚠ CRITICAL: Avoid freeze-thaw cycles of cytokine solution to maintain activity.

- 1× Red Blood Cell (RBC) Lysis Buffer: Add 0.5 mL 10× RBC Lysis Buffer to 4.5 mL distilled water.

Store 10× RBC Lysis Buffer at 4°C and use within 6 months. Prepare 1× buffer fresh before using.

Alternatives: ACK (ammonium-chloride-potassium) lysing buffer.

Store at ~23°C for up to 6 months.

- 2% PFA solution: Add 10 mL 16% paraformaldehyde (PFA) into 70 mL sterile DPBS.

Store at 4°C in the dark and use within 1 month.

⚠ CRITICAL: PFA is a chemical hazard. Use with caution.

- Citric acid-Na_2HPO_4 Buffer (PH = 6.0): Mix 36.85 mL 0.1 M citric acid with 63.15 mL 0.2 M Na_2HPO_4, confirm PH is 6.

Keep at ~23°C for 3 months, check PH before using.

- X-Gal stock solution (40 mg/mL): Dissolve 1 g X-Gal into 25 mL dimethylformamide (DMF).

Distribute into 1 mL aliquots and store at −20°C for up to 1 year.

⚠ CRITICAL: DMF is a chemical hazard. Use with caution.

STEP-BY-STEP METHOD DETAILS

Generation of bone marrow derived CD103$^+$ dendritic cells (BMDCs)

Timing: 10–14 days

The following section describes the isolation of mouse bone marrow cells, as well as the differentiation and characterization of CD11c$^+$/CD103$^+$ DCs based on a previous publication.[16] While the yield may vary between mice, we usually obtain 40–60 million cells from both femurs of a 7–9-week-old female BALB/c mouse. Freshly isolated mouse bone marrow cells are differentiated with FMS-like tyrosine kinase 3 ligand (Flt3 ligand) and granulocyte-macrophage colony-stimulating factor (GM-CSF), then phenotypically characterized using flow cytometry for CD11c$^+$/CD103$^+$ DCs expressing major histocompatibility complex (MHC) molecules.

1. Obtain femurs from a 7–9-week BALB/c mouse.
   a. Spray mouse surface with 70% ethanol after it has been euthanized according to the institution's protocol.
   b. Dissect out both femurs without breaking the epiphyses, and carefully remove the muscle and fibrous tissues without damaging the bones. Use a Kimwipe saturated with 70% ethanol to remove excess muscle if necessary.

⚠ CRITICAL: The bone must remain intact in order to obtain non-contaminated bone marrow cells with high viability.

Note: Typically, we begin by removing the soft tissue surrounding the femur to expose the epiphysis as the starting point for dissection. The femur is then dissected from the epiphysis using a sharp and fine dissection scissor. It is critical to process gently and carefully to avoid excessive pressure or force that damages bones. Maintaining an intact bone can greatly help in preventing bone marrow contamination and preserving cell viability.

   c. Place the bones in a 1.5 mL Eppendorf tube containing 1 mL of ice-cold sterile PBS. Transport on ice.

Note: If more bone marrow is needed, other long bones, such as the tibia and humerus, can be harvested from the same mouse for bone marrow isolation.

2. Collect bone marrow cells from the femur. The following steps should be performed under a tissue culture hood to avoid contamination.
   a. Rinse the bones in 70% ethanol, then wash them in ice-cold sterile PBS to remove ethanol. Transfer the bones into a sterile Petri dish that contains 7 mL of basic immune cell medium.
   b. Connect a 25-gauge needle to a 5 mL syringe filled with basic immune cell medium.
   c. Cut off both epiphyses using sterile scissors. Hold the bone with forceps while flushing the marrow into the Petri dish.

Note: Flush the bone from both ends and scrape its inner surfaces with needle as needed. 5 mL of media is usually sufficient to flush all the bone marrow from one femur. If necessary, repeat the flushing process until the bone appears pale. The change in color of the femur from red to pale indicates that the bone marrow has been fully removed.

   d. Transfer all cells from the Petri dish through a 40 μm strainer into a 50 mL Falcon tube.
   e. Wash the Petri dish and strainer twice with 7 mL basic immune cell medium to collect the remaining cells. Discard the strainer and cap the Falcon tube with cells.
   f. Centrifuge cells at 300 × *g* for 5 min and discard the supernatant.

Note: In this protocol, we used an Eppendorf benchtop centrifuge with a swinging-bucket rotor that accommodates 50/15 mL Falcon tubes for cell pelleting. Alternatively, depending on the available laboratory equipment, other centrifuge models may also be utilized.

Note: The cell pellets are currently red.

g. Resuspend the cells in 5 mL of 1× Red Blood Cell (RBC) Lysis Buffer and incubate on ice for 5–10 min with occasional shaking.
h. Add 20–30 mL of basic immune cell medium to stop the reaction.
i. Pellet cells by centrifuging at 300 × *g* for 5 min. Discard the supernatant.

Note: White cell pellets indicate sufficient lysis of red blood cells.

j. Resuspend the cells in 10 mL DC medium. Count cells and adjust to 2×10^6 viable cells/mL. Troubleshooting 1.

Note: Cell viability is expected to be >90%.

Note: For cell counting, we typically mix 10 μL cell suspension and 10 μL trypan blue to stain and exclude dead cells. Load 10 μL of this mixture onto a hemocytometer for manual counting. Alternatively, automated cell counters that distinguish live and dead cells can be used.

3. Differentiate bone marrow cells into $CD11c^+/CD103^+$ DCs in the presence of Flt3 ligand (200 ng/mL) and GM-CSF (1 ng/mL).
   a. Transfer the cell suspension from the Falcon tube into a T75 flask, then place the flask into a regular cell culture incubator.

   Note: We typically get 20–30 mL of cell suspension containing 2×10^6 cells/mL from one mouse. This results in 40–60 million cells in total, which can be maintained in a T75 flask. If more cells are collected, divide them into several flasks based on the final volume. Conversely, if the cell count is low, resuspend the cells to 2×10^6 cells/mL and transfer them to a smaller flask (e.g., T25) aligned with the final volume.

   b. On Day 6, transfer half of the cell suspension (10–15 mL) into a sterile Falcon tube. Centrifuge cells at 300 × *g* for 5 min and discard the supernatant.
   c. Resuspend the cell pellets in the same amount of fresh DC medium (10–15 mL) and place them back in the old flask.
   d. On Day 9, gently tap the flask and harvest all non-adherent cells, then count the cell number.

   Note: DCs generated here using GM-CSF and Flt3 ligand are primarily non-adherent cells.

   e. Based on the cell count, calculate the total volume of cell suspension required to achieve a target density of 3×10^6 viable cells/mL.

   Note: We typically obtain a concentration of roughly 1–2.5 × 0^6 cells/mL from this step. Using a concentration of 2×10^6 cells/mL in 30 mL as an example, concentrating the suspension to 20 mL would be required to achieve 3×10^6 cells/mL.

   f. Centrifuge the cells at 300 × *g* for 5 min. Remove sufficient supernatant to leave the cells at twice the target density.
   g. Dilute the DCs to 3×10^6 cells/mL by adding an equal volume of fresh DC medium and resuspend the cell pellet by gently pipetting. Transfer the entire cell suspension to a new T75 flask and return it to the incubator.

Note: If the cell count is low, adjust the suspension to 2–3 × 10^6 viable cells/mL and move the cells to a smaller flask (like a T25) that corresponds to the final volume.

Note: Handle the cells gently to prevent mechanical stress.

Note: From Day 10, $CD11c^+/CD103^+$ DCs can be identified and used for coculture assays, although their percentages may be low. Extending the incubation time to 14 days usually leads to a higher percentage of $CD11c^+/CD103^+$ DCs.

h. Perform another DC medium change on Day 12 as described above.

4. Phenotype $CD103^+$ DCs on Day 14.
   a. Examine DC morphology under a phase contrast microscope prior to harvesting. Typically, $CD103^+$ DCs display strongly branched dendrites.
   b. Gently tap the flask and transfer 2 mL of cell suspension into a FACS tube.

Note: $CD103^+$ DCs usually remain suspended individually or cluster together by Day 14.

c. Count cells and make an aliquot of 3×10^5 viable cells for surface staining in a new FACS tube.

Note: We typically obtain approximately 1 × 10^6 viable cells (above 3 × 10^5) from a 2 mL cell suspension. If the cell counts fall below this or if additional cells are required for further characterization or other experiments, a larger volume of cell suspension could be collected.

d. Add 1 mL PBS to the FACS tube with 3×10^5 viable cells. Centrifuge cells at 300 × *g* for 5 min and discard the supernatant.
e. Resuspend cell pellets in 100 µL PBS containing Zombie Yellow diluted at 1:2000 and incubate for 15 min at ~23°C in the dark.

Optional: Add 0.5 µg anti-mouse CD16/32 antibody to the Zombie Yellow live/dead staining buffer to block Fc receptors.

f. To wash the cells, add 2 mL of Cell Staining Buffer, centrifuge at 300 × *g* for 5 min, and discard the supernatant.
g. Stain the surface proteins with the following antibody panel in a final volume of 100 µL. Incubate at 4°C for 30 min in the dark.

Reagent	Final dilution ratio	Amount
Pacific Blue anti-mouse CD11c Antibody	1:200	0.5 µL
Brilliant Violet 711 anti-mouse CD103 Antibody	1:400	0.25 µL
Alexa Fluor 488 anti-mouse H-$2K^d$ Antibody	1: 200	0.5 µL
APC/Cyanine7 anti-mouse I-A/I-E Antibody	1: 200	0.5 µL
Brilliant Stain Buffer	10%	10 µL
Cell Staining Buffer	N/A	87.75 µL
Total	N/A	100 µL

Note: Preparing a single stained control for compensation using cells or UltraComp eBeads is recommended.

Note: Adding Brilliant Stain Buffer may improve staining results when using antibodies conjugated with BD Horizon Brilliant dyes, including Brilliant Violet 711.

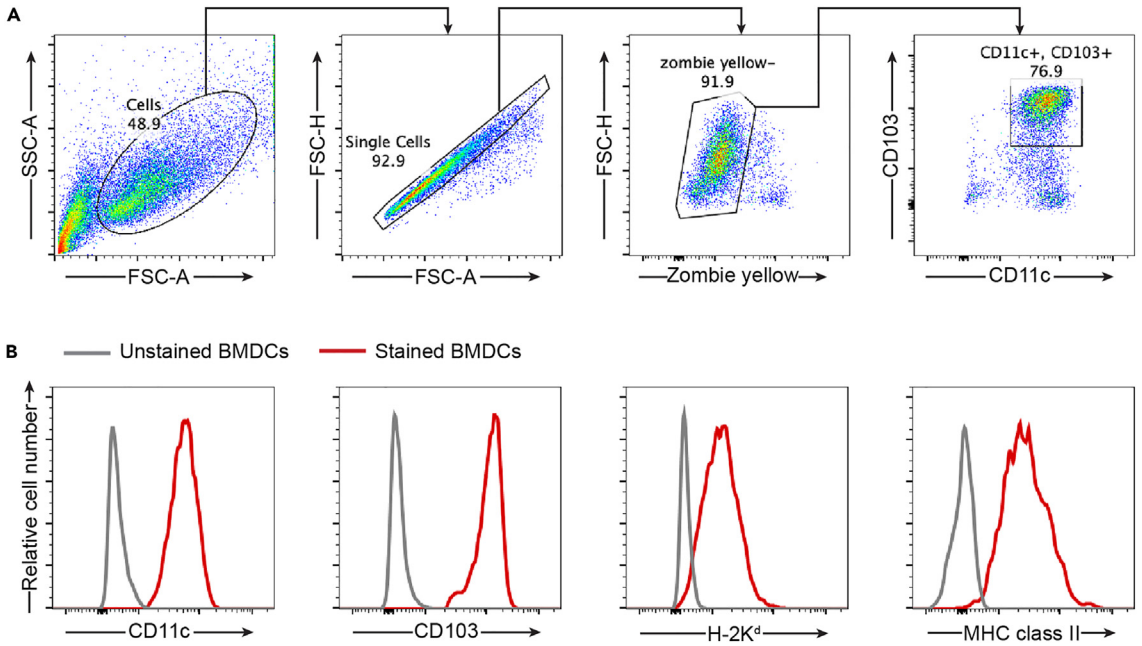

Figure 1. Phenotypic characterization of bone marrow-derived dendritic cells (BMDCs) generated using GM-CSF and Flt3 ligand

Bone marrow cells were cultured with GM-CSF (1 ng/mL) and Flt3 ligand (200 ng/mL) for 14 days before harvesting for flow cytometry analysis. A, Flow cytometry gating strategy for BMDCs. Cells were first gated based on forward scatter (FSC) and side scatter (SSC), then gated for single live cells by size (FSC-A and FSC-H) and Zombie yellow exclusion. To identify DCs, the single viable cells were gated on co-expression of CD11c and CD103. B, Representative histograms displaying the surface expression levels of CD11c, CD103, class I MHC molecule H-2K^d, and class II MHC molecules I-A/I-E in CD11c$^+$/CD103$^+$ BMDCs. Gray lines indicate unstained controls, while red lines represent stained BMDCs.

h. Wash samples with 1 mL of Cell Staining Buffer, then centrifuge at 300 × *g* for 5 min and discard the supernatant. Resuspend cell pellets in 300 μL Cell Staining Buffer and analyze by flow cytometry. Figure 1 shows the representative phenotypic profile of CD11c$^+$/CD103$^+$ DCs. Troubleshooting 2.

Pause point: Freshly stained cells can be kept at 4°C in the dark for up to 2 h before analysis. Alternatively, cells can be fixed in 2% PFA for 15 min at ~23°C, followed by one wash with PBS and resuspension in 300 μL of Cell Staining Buffer. The fixed cells should be stored at 4°C in the dark for up to a week before analysis.

Note: The percentage of CD11c$^+$/CD103$^+$ DCs in the single live cell population is expected to reach 70% on Day 14.

In vitro preparation of therapy-induced senescent tumor cells

Timing: 5 days

Multiple reagents have been found to induce senescence in tumor cells.[17] Here, we utilize the topoisomerase poison etoposide (2 μM) and the Polo-like kinase 1 (PLK-1) inhibitor GSK461364 (2 μM) to trigger senescence in the murine colon carcinoma cell line CT26. Senescent cells (SnCs) are often characterized by the upregulation of lysosomal β-galactosidase, also known as senescence-associated β-galactosidase (SA-β-Gal),[5] which can be detected through the SA-β-Gal staining assays.[18] Among various SA-β-Gal substrates, one of the most widely used is X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which produces a blue precipitate when cleaved, allowing visualization under a microscope.[19] This section describes the protocol for evaluating tumor cell senescence

using X-Gal *in vitro*. The senescence inducers used here have been successfully applied to other mouse tumor cell lines, including murine mammary carcinoma 4T1 and melanoma B16. It is important to note that this protocol may require optimization when using alternative senescence inducers or different cell lines.

5. Maintain CT26 cells.
   a. Prewarm complete RPMI medium, PBS, and trypsin-EDTA in a 37°C bead bath.
   b. Passage cells once they have reached 80%–90% confluence.
      i. Remove the cell culture medium and rinse the cells once with PBS.
      ii. Add 4 mL of trypsin-EDTA to the T75 flask and rock gently to completely cover the cells.
      iii. Incubate the flask at 37°C for approximately 2 min.
      iv. Once the cells have detached, add 8 mL of pre-warmed complete RPMI medium and disperse the cells by gently pipetting.

   Note: If working with cell lines sensitive to trypsin-EDTA, pellet the cells by centrifuging at 300 × *g* for 5 min. Following that, discard the supernatant to remove residual trypsin-EDTA, and resuspend the cells in complete RPMI medium.

      v. Transfer 1/4–1/8 volumes of the cell suspension to a new T75 flask and add 15 mL of complete RPMI medium. Return the cells to the incubator.

   Note: Performing experiments on cells within ten passages is recommended.

6. Induce senescence using etoposide or GSK461364.
   a. Seed 2.5×10^4 CT26 cells with 1 mL medium in each well of a 12-well plate and allow them to attach overnight (12–16 h).

   Note: If using a different-sized cell culture plate, adjust the cell seeding density according to the well's surface area.

   b. Add etoposide or GSK461364 to a final concentration of 2 μM. An equal amount of DMSO vehicle should be applied to the control cells.

   Note: Consider reducing the incubation time for non-senescent control cells to avoid overgrowth.

   c. Culture the cells in the presence of senescence inducers for 5 days.

   Note: If other cell lines or senescence inducers are used, cell seeding density, senescence inducer dose, and senescence induction timing may need to be optimized. Ideally, both the SnCs and the non-senescent controls should achieve a confluency of 70%–80% by the time of SA-β-Gal staining.

   Note: It is critical to seed the cells at an appropriate density for senescence induction. In our experience, we aim for 30%–50% confluence at the time of drug treatment. This prevents cells from overgrowing prior to entering senescence. We have noticed that cells at high density often exhibit reduced responsiveness to senescence inducers.

   Note: For senescence induction, we usually do not change the medium during the 5-day incubation period. In case the medium needs to be supplemented/replaced due to evaporation, ensure that the added medium contains the same concentration of senescence inducers or DMSO vehicle.

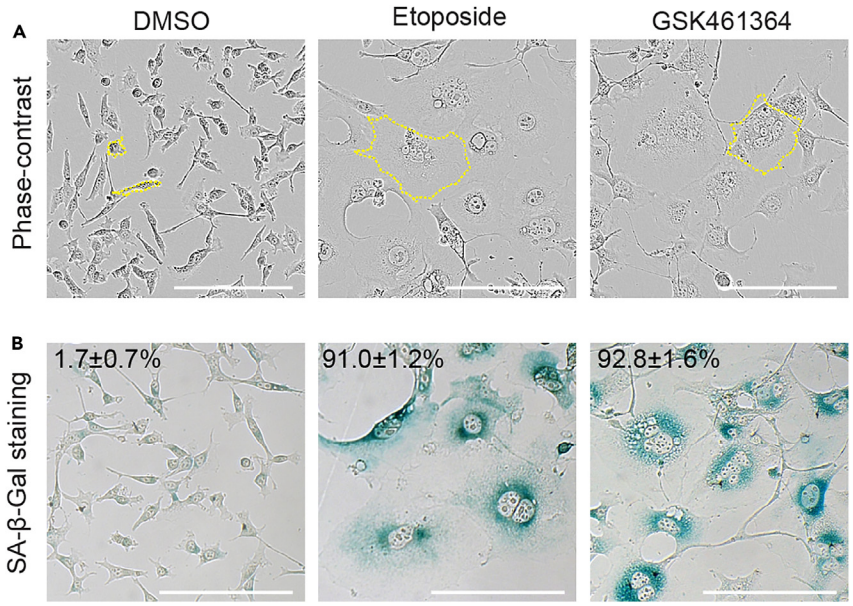

Figure 2. Senescence induction in CT26 cells

Cellular senescence was induced by etoposide (2 μM) or GSK461364 (2 μM) for 5 days. CT26 cells treated with DMSO vehicle were used as controls. A, Representative phase-contrast images. Cell contours on bright-field images are indicated by yellow dotted lines. Scale bars: 200 μm. B, SA-β-Gal staining of CT26 cells. The mean ± SD percentage of SA-β-Gal-positive cells from five 20× fields is indicated. Scale bars: 100 μm.

7. Characterize senescence using X-Gal.
   a. Before SA-β-Gal staining, examine the cell morphology under a phase contrast microscope as SnCs typically adopt a flat, enlarged cell morphology (Figure 2A).

   Note: For SA-β-Gal staining, a cell confluency between 70%–80% is optimal. While a lower confluency is acceptable, exceeding it may lead to false positive staining results.

   b. Remove the cell culture medium and gently wash the cells once with PBS.
   c. Fix cells in 2% PFA for 3–5 min.

   ⚠ CRITICAL: Fixation beyond 5 min may inhibit SA-β-Gal activity and cause false negative staining results.

   d. Stain the cells overnight (12–16 h) at 37°C with SA-β-Gal staining solution as follows:

Reagent	Final concentration	Amount
Citric acid-Na_2HPO_4 Buffer (PH = 6.0)	N/A	10 mL
Sodium chloride (1.5 M)	150 mM	5 mL
Magnesium chloride (20 mM)	2 mM	5 mL
Potassium ferricyanide (500 mM)	5 mM	0.5 mL
Potassium ferrocyanide (500 mM)	5 mM	0.5 mL
X-Gal (40 mg/mL dissolved in DMF)	1 mg/mL	1.25 mL
H_2O	N/A	27.75 mL
Total	N/A	50 mL

Note: Prewarm the SA-β-Gal staining solution to 37°C and filter it through a 0.2 μm syringe filter to remove X-Gal precipitate before applying to cells.

Note: We typically add 1 mL of SA-β-Gal staining solution to each well of a 12-well plate. If a different-sized cell culture plate is used, adjust the staining solution volume accordingly.

Note: The incubation time may need to be optimized for different cell lines.

e. Gently wash the cells twice with PBS prewarmed to 37°C. Keep the stained cells in PBS for imaging under a microscope with a color camera. Troubleshooting 3.

Note: In our experience, treatment with 2 μM etoposide or GSK461364 can result in over 90% SA-β-Gal$^+$ senescent CT26 cells (Figure 2B).

Pause point: Stained cells can be kept at 4°C in the dark for up to two weeks before imaging. Alternatively, stained cells can be stored in 70% glycerol in the dark at 4°C for longer.

Coculturing of senescent CT26 cells and BMDCs

Timing: 1–2 days

In this session, fresh CD103$^+$ DCs are incubated with senescent tumor cells overnight at an approximate ratio of 2:1. In this example, we used senescent CT26 cells induced by etoposide or GSK461364 on Day 5, along with BMDCs collected on Day 14. If different cell lines or senescence inducers are used, the optimal protocol for senescence induction needs to be determined beforehand. This protocol has been successfully applied to other BALB/c-derived cell lines, including 4T1. When working with C57BL/6 derived tumor cell lines, such as B16 melanoma cells, it is recommended to obtain bone marrow from C57BL/6 mice. Matching the haplotype is particularly critical if the BMDCs are to be utilized for functional assays, such as T cell priming.

8. Maintain senescent tumor cells in basic immune cell medium after removing senescence inducers.
   a. Induce CT26 cell senescence as described above. Cells treated with DMSO vehicle are recommended as non-senescent controls. Cells should be prepared at least in duplicate.

   Note: Non-senescent cells should not be allowed to overgrow when used as controls, potentially requiring shorter incubation times.

   b. Examine the cell morphology under a microscope before proceeding. Verify that CT26 cells have developed a typical enlarged senescent morphology without too many dead cells.
   c. For one of the duplicate samples, wash the cells twice with 2 mL sterile PBS to remove any small molecule residue, while collecting cells from the other sample for cell counting.

   Note: Counting senescent or non-senescent tumor cells is helpful for determining the appropriate number of BMDCs needed for coculture experiments.

   d. Replace PBS with 1.5 mL fresh basic immune cell medium and return cells to the incubator.

   Note: SnCs can be maintained in basic immune cell medium for 4 h–16 h before coculturing with BMDCs. In this medium, senescent cells remain viable, while non-senescent controls continue to proliferate.

   Note: The primary objective of this step is to allow the accumulation of SASP factors in the medium. While we generally keep the senescent cells in the basic immune cell medium for 16 h before coculture with BMDCs, it is noteworthy that BMDC activation has been observed when

etoposide-induced senescent cells are preserved in the medium for just 4 h before coculture. The time required to incubate SnCs in basic immune cell medium before coculture may vary depending on their immunogenicity.

9. Coculture BMDCs with SnCs.
   a. Collect DCs in sterile tubes and centrifuge them for 5 min at 300 × *g*, then decant the medium.
   b. Resuspend DCs in fresh immune cell medium.
   c. Count viable cells and adjust the concentration to 0.5×10^6 viable cells/mL.
   d. Add BMDCs to SnCs at an approximate ratio of 2:1.

   Note: The number of BMDCs required for each experiment may vary depending on the numbers of senescent and non-senescent cells. As mentioned above, we typically culture adherent SnCs in a 12-well plate, producing approximately 0.5×10^5 SnCs per well. Given that, we usually introduce around 1×10^5 BMDCs in 200 μL per well into the 12-well plate for coculture experiments. Adjust the BMDC count accordingly if different plate types are used. The same 2:1 ratio should be maintained when coculturing BMDCs with non-senescent control cells. As highlighted earlier, ensure that non-senescent controls do not overgrow before coculture. It's also worth noting that slight deviations from the 2:1 ratio will not affect BMDC responses.

   e. Return cells to the incubator and culture overnight (12–16 h).

   Note: During coculture, the medium can be supplemented with GM-CSF and Flt3 ligand at final concentrations of 0.2 ng/mL and 40 ng/mL, respectively, to boost the viability of BMDCs.

   Note: During coculture, the BMDCs are exposed to the SASP, come in contact with SnCs, and take up SnC membranes and cytoplasm via phagocytosis or trogocytosis, each of which may influence DC phenotypes. We have observed that DC activation can occur as soon as 10 h after exposure to etoposide induced CT26 SnCs. The time required to detect DC activation triggered by SnCs may vary depending on the senescence induction methods and the immunogenicity of the SnCs.

   Note: It is important to incorporate appropriate controls to enable reliable assessment of BMDC activation and maturation. Unstimulated BMDCs may be used as a negative control while stimulation with lipopolysaccharide (LPS, 2 μg/mL) can serve as a positive control.[20]

Characterization of BMDC maturation and activation

Timing: 4 h

To assess the activation and maturation of $CD103^+$ BMDCs, the expression levels of various surface markers can be evaluated through flow cytometry. Here, these markers include the upregulation of co-stimulatory factors CD80 and CD86, as well as the class I MHC molecule $H\text{-}2K^d$. CD80 and CD86 play critical roles in initiating and sustaining T cell responses by interacting with CD28 and potentially other unidentified receptors on T cell surface. MHC class I molecules present antigens to $CD8^+$ T cells and regulate T cell activation and proliferation through TCR signals.[21] We also examine PD-L1 expression on the DC surface, which dampens T cell activity.[22] This is because PD-L1 is upregulated by a variety of stimuli,[23] including Type I interferons (IFNs) and multiple pro-inflammatory cytokines that can be secreted by SnCs as components of the SASP.[24] It is noteworthy that class I MHC molecules expressing haplotype b, such as $H\text{-}2K^b$, should be analyzed when using DCs derived from the bone marrow of C57BL/6 mice.

10. Harvest BMDCs into FACS tubes.
    a. Gently pipette the cocultured cells up and down, then transfer all the suspended cells to a FACS tube.
    b. Rinse the well with 1 mL of PBS to collect the remaining cells.

Note: In the cocultures, the SnCs typically remain adherent while the BMDCs can be readily suspended, allowing them to be collected selectively simply by swirling and pipetting.

    c. Pellet the cells at 300 × *g* for 5 min, then decant the supernatant.
11. Stain the BMDCs for flow cytometric analysis. Troubleshooting 4 and 5.
    a. Resuspend cell pellets in 100 μL PBS containing Zombie Yellow diluted at 1:2000 and 0.5 μg anti-mouse CD16/32 antibody, then incubate for 15 min at ~23°C in the dark.
    b. Add 2 mL of Cell Staining Buffer to the FACS tube to wash the cells, followed by centrifuging at 300 × *g* for 5 min and discarding the supernatant.
    c. Prepare the surface protein staining solution as follows. Add 100 μL of staining solution to each sample and incubate at 4°C for 30 min in the dark.

Reagent	Dilution ratio (1×)	Amount
Pacific Blue anti-mouse CD11c Antibody	1:200	5 μL
Brilliant Violet 711 anti-mouse CD103 Antibody	1:400	2.5 μL
PE/Cyanine7 anti-mouse CD86 Antibody	1: 200	5 μL
APC anti-mouse CD80 Antibody	1: 200	5 μL
Alexa Fluor 488 anti-mouse H-2K^{d} Antibody	1: 200	5 μL
Brilliant Stain Buffer	10%	100 μL
Cell Staining Buffer	N/A	877.5 μL
Total	N/A	1000 μL

Note: Preparing single stained control samples for compensation is recommended.

Note: If additional activation markers are investigated, it is necessary to titrate the corresponding antibodies and ensure the flow cytometry panel is compatible. DCs stimulated by LPS can be used to evaluate antibody performance, especially for activation markers.

    d. For washing cells, add 2 mL of Cell Staining Buffer to the FACS tube and centrifuge at 300 × *g* for 5 min. Carefully discard the supernatant.
    e. Resuspend cell pellets in 300 μL Cell Staining Buffer and analyze the cells by flow cytometry.

Note: Freshly stained BMDCs can be kept on ice or at 4°C for up to 2 h before analysis. Alternatively, cells can be fixed in 2% PFA for 15 min at ~23°C and kept at 4°C for future analysis.

Note: For our flow cytometry experiments, we utilized a BD LSR Fortessa Cell Analyzer. This analyzer also provides a high throughput mode compatible with 96-well plates. While we typically collect, stain, and analyze samples using FACS tubes, 96-well round bottom polystyrene plates can also be utilized when a plate-based approach is preferred.

EXPECTED OUTCOMES

This protocol is designed to examine the potential for senescent tumor cells to stimulate dendritic cells (DCs), providing a rapid method for determining the immunogenicity of cellular senescence. As shown in Figure 3, DC maturation and activation are characterized by the increased surface expression of costimulatory molecules CD80 and CD86, and class I MHC molecule H-2K^{d}. The PD-L1 level is also upregulated when DCs are stimulated, as shown by changes in both population percentage

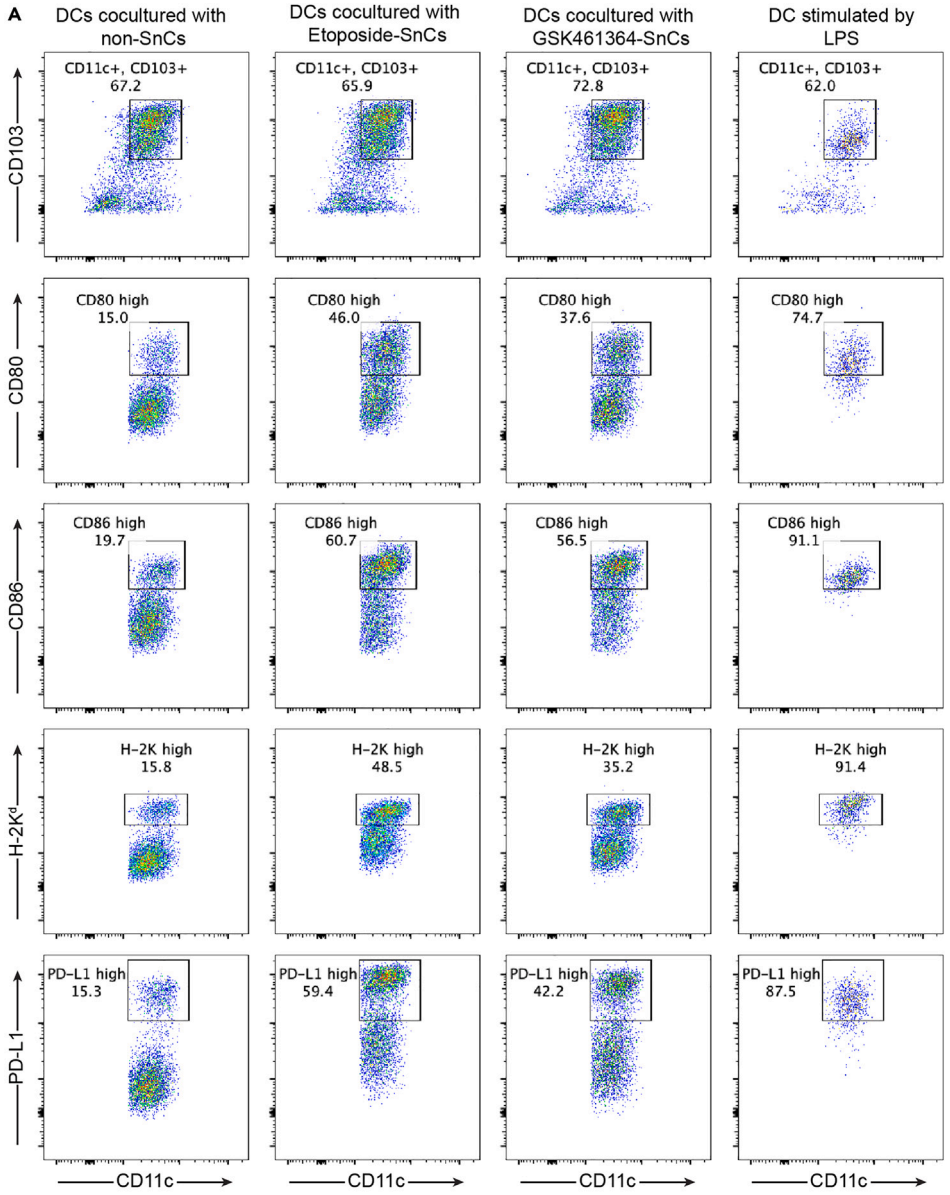
A
DCs cocultured with non-SnCs
DCs cocultured with Etoposide-SnCs
DCs cocultured with GSK461364-SnCs
DC stimulated by LPS
CD11c+, CD103+ 67.2
CD11c+, CD103+ 65.9
CD11c+, CD103+ 72.8
CD11c+, CD103+ 62.0
CD103
CD80 high 15.0
CD80 high 46.0
CD80 high 37.6
CD80 high 74.7
CD80
CD86 high 19.7
CD86 high 60.7
CD86 high 56.5
CD86 high 91.1
CD86
H-2K high 15.8
H-2K high 48.5
H-2K high 35.2
H-2K high 91.4
H-2Kd
PD-L1 high 15.3
PD-L1 high 59.4
PD-L1 high 42.2
PD-L1 high 87.5
PD-L1
CD11c
B
DC cocultured with:
DMSO non-SnCs
Etoposide SnCs
GSK461364 SnCs
Geometric MFI of CD80
Geometric MFI of CD86
Geometric MFI of H-2Kd
Geometric MFI of PD-L1
***

Figure 3. Senescent cells promote DC maturation and activation *in vitro*

BMDCs were cultured overnight with CT26 SnCs and control cells, then stained for $CD11c^+$, $CD103^+$, CD80, CD86, $H\text{-}2K^d$, and PD-L1 and analyzed by flow cytometry. LPS-activated DCs served as positive controls. A, Representative dot plots showing $CD11c^+/CD103^+$ DCs in a single viable cell population after coculture, along with CD80, CD86, $H\text{-}2K^d$, and PD-L1 surface expression levels in gated $CD11c^+/CD103^+$ DC population. B, For quantitative analysis of DC activation/maturation, the mean fluorescence intensity (MFI) of CD80, CD86, $H\text{-}2K^d$, and PD-L1 was determined in $CD11c^+/CD103^+$ DC population. Note distinct patterns of activation by etoposide- and GSK461364-induced SnCs, particularly with respect to PD-L1 expression. Data from three experiments, mean ± SD. ***$p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$ (unpaired t-test).

(Figure 3A) and mean fluorescence intensity (MFI) (Figure 3B). Notably, the capability of senescent cells to activate DCs varies depending on the specific senescence inducers. Results of this assay may provide insight into the interaction between different methods of senescence induction and corresponding immune responses.

QUANTIFICATION AND STATISTICAL ANALYSIS

FlowJo version 10 (FlowJo, LLC https://www.flowjo.com/) was used for data analysis. It is recommended to perform at least three independent replicates for statistical analysis. Statistical significance was determined using the unpaired Student's t-test. Calculations were performed using Prism software (GraphPad). $p \leq 0.05$ was considered statistically significant.

LIMITATIONS

In the current protocol, upregulated surface markers are used as indicators of dendritic cell activation. However, these markers may not necessarily reflect DC antigen-presenting (APC) capabilities. To evaluate the APC function, additional assays, such as T cell priming experiments using DCs stimulated by senescent cells, may be required.

Due to the complexity of the immune system, which includes various cell types, the impact of therapy-induced senescence on DCs *in vitro* may not be a reliable indicator of their potential to influence immune responses in the tumor microenvironment. A series of sophisticated assays is necessary to examine whether the senescent tumors cells play beneficial or detrimental roles in anti-cancer therapy responses *in vivo*.

TROUBLESHOOTING

Problem 1

Low viability of bone marrow cells.

Potential solution

Keep the bones moist during the transfer and processing. Verify that the joints are intact before dipping the bones in ethanol. Make sure the bones are not left in ethanol for too long. Additionally, watch the time closely during red blood cell lysis, as excess time in the lysis solution could kill other cells.

Problem 2

Low yield of $CD11c^+/CD103^+$ DCs.

Potential solution

Keep cytokines in proper storage to maintain their stability. Thawing reconstituted cytokines repeatedly can lead to loss of activity. Different batches or lots of cytokines may affect DC differentiation duration. It is recommended to test cytokines from a new lot each time to determine the optimal time window for differentiation. In our experience, DC differentiation using GM-CSF and Flt3 ligand is best achieved between Day 10 and 14. Culturing bone marrow-derived dendritic cells (BMDCs) for extended periods may affect their viability and activation.

Problem 3

Low yield of senescent cells or low percentage of SA-β-Gal$^+$ cells.

Potential solution

Senescence induction methods need to be optimized for each cell line under study. Many small molecule senescence inducers are cytotoxic. Thus, it is recommended to titrate their concentrations to achieve the most effective senescence induction, balancing cell cycle arrest and cell viability. The duration of the senescence induction process can vary based on the type of cells and the induction methods. Cells should be consistently cultured under optimal conditions. Stress-inducing conditions, including excessive passaging, suboptimal medium, or high cell density, can negatively affect cell health and their response to stress, including senescence inducers.

Regarding SA-β-Gal staining, it is important to maintain the staining buffer pH at 6 and incubate cells in a non-CO_2 incubator. A high pH may reduce the effectiveness of staining. Although most of our tested cell lines require prolonged staining over several hours, the staining duration needed may vary for different cell lines. In addition, over-fixing cells in PFA may affect SA-β-Gal activity, resulting in false negative staining results.[25]

Problem 4

No DC activation observed.

Potential solution

Unresponsive DCs might be dead or unhealthy. Check the viability and health of BMDCs before co-culturing them with senescent cells. To prevent damage to DCs, handle them gently throughout the process. For confirmation of DC activation capability, LPS stimulation can be used as a positive control. In our experience, DC activation can occur between 10 and 16 h after coculture with senescent cells. However, the required time for DC activation may vary depending on the type of senescent cells or preparation of DCs.

Problem 5

High expression of activation markers on unstimulated DCs.

Potential solution

Establish optimal culture conditions for BMDCs before initiating coculture experiments and maintain sterile conditions throughout all tissue culture experiments. DCs may be activated during culture through stresses caused by suboptimal conditions, such as high cell density, inappropriate culture medium, prolonged culture with GM-CSF and Flt3 ligand, contamination, or other exposure to inflammatory factors. During passaging, harvesting, or washing, handle cells gently throughout the entire process to avoid upregulation of activation markers due to mechanical stress.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen J. Kron (skron@uchicago.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This study did not generate datasets/code.
- Original/source data reported in the paper is available from the lead contact upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.L. and S.J.K.; methodology, Y.L.; writing – review and editing, Y.L. and S.J.K.; funding acquisition, S.J.K.; supervision, S.J.K.; and project administration, S.J.K.

DECLARATION OF INTERESTS

S.J.K. is a co-founder of OncoSenescence and Riptide Therapeutics. Y.L. is currently employed by Calico Life Sciences LLC.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used Chat GPT-4 in order to improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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