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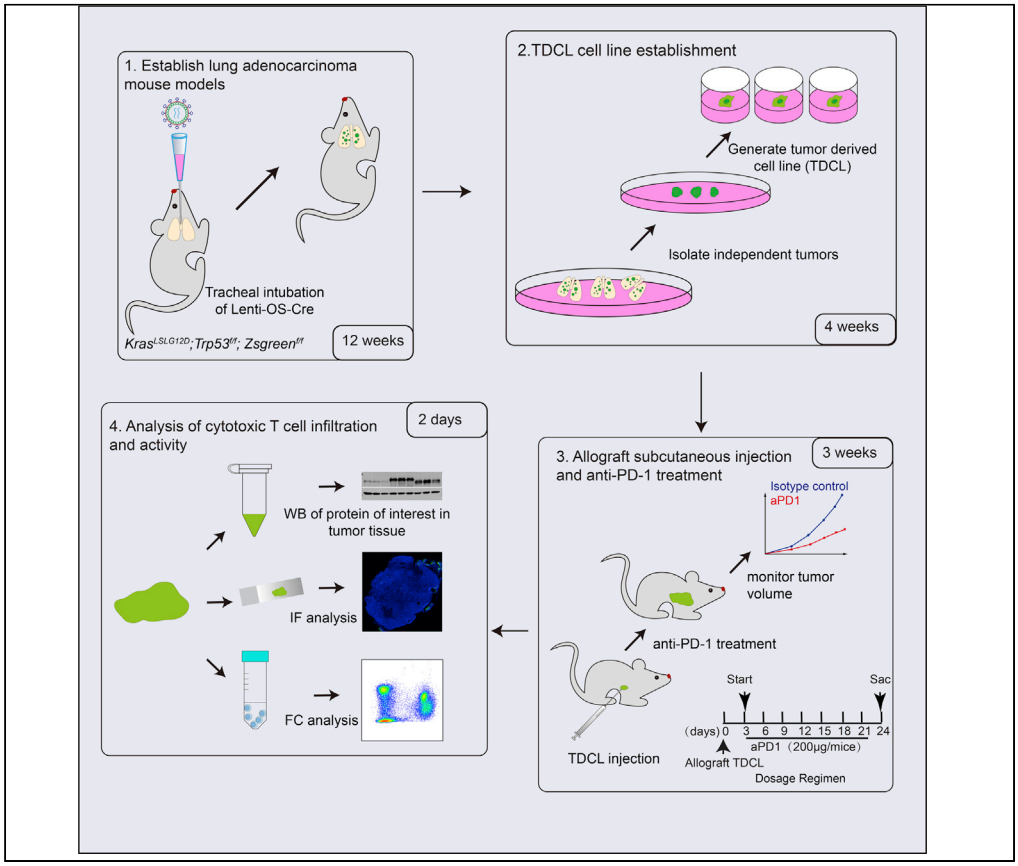
Protocol to establish a lung adenocarcinoma immunotherapy allograft mouse model with FACS and immunofluorescence-based analysis of tumor response



Anti-PD-1/PD-L1 therapy shows long-term effects in many cancer types, but resistance and relapse remain the main limitations of this therapy. Here, we describe a protocol to evaluate the tumor response to immunotherapy in a mouse lung cancer model. The protocol includes the establishment of the lung cancer mouse model, anti-PD-1 treatment, tumor-infiltrating lymphocyte isolation, immunofluorescence, and flow cytometry analysis. This protocol can also be applied to other cancer types and immunotherapies.

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Highlights

Establish a tumor-derived cell line from a genetic engineered lung cancer mouse model

Evaluate the tumor response to immunotherapy using an allograft lung cancer model

Isolate tumor-infiltrating lymphocytes from fresh tumor samples

Evaluate activity of lymphocytes by flow cytometry and infiltration by immunofluorescence

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Protocol to establish a lung adenocarcinoma immunotherapy allograft mouse model with FACS and immunofluorescence-based analysis of tumor response

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SUMMARY

Anti-PD-1/PD-L1 therapy shows long-term effects in many cancer types, but resistance and relapse remain the main limitations of this therapy. Here, we describe a protocol to evaluate the tumor response to immunotherapy in a mouse lung cancer model. The protocol includes the establishment of the lung cancer mouse model, anti-PD-1 treatment, tumor-infiltrating lymphocyte isolation, immunofluorescence, and flow cytometry analysis. This protocol can also be applied to other cancer types and immunotherapies.

For complete details on the use and execution of this protocol, please refer to Yu et al. (2021)

BEFORE YOU BEGIN

$Kras^{Lox\text{-}STOP\text{-}Lox(LSL)\text{-}G12D}$; $Trp53^{flox/flox}$; $ZsGreen^{flox/flox}$ (KPZ) mouse preparation

Timing: 12 weeks

Prepare 10 *KPZ male* mice at 6–8 weeks of age.

1. Perform mouse crossing and breeding.
2. Purchase the Kras P53, and Zsgreen mice from Jax lab.
   a. KPZ mice model is established by crossing $Kras^{Lox\text{-}STOP\text{-}Lox(LSL)\text{-}G12D}$, $Trp53^{flox/flox}$ and $ZsGreen^{flox/flox}$ mice. Finally, the mice is heterozygous for $Kras^{Lox\text{-}STOP\text{-}Lox(LSL)\text{-}G12D}$ and homozygous for $Trp53^{flox/flox}$ and $ZsGreen^{flox/flox}$.
   b. Expand 6v8 week-old male and female KPZ mice as the parents.
   c. F1 offsprings can be generated approximately 4 weeks later.
3. Perform genotyping.
   a. Tail lysis.
      i. Add 200 μL 1× mouse tissue lysis buffer and 4 μL 10 mg/mL Proteinase K (Vazyme, CAT PD101-01) to the tail and incubate at 55°C for 12h
      ii. Incubate at 95°C for 5 min and collect the supernatant by centrifugation at 140000 × *g* for 5 min at 25°C.
   b. Primers for Kras genotyping (from the Jackson Laboratory).

Primer name	Sequence 5′-3′
K-RasG12D 22907	TGTCTTTCCCCAGCACAGT
K-RasG12D 22908	CTGCATAGTACGCTATACCCTGT
K-RasG12DLSL oIMR9592	GCAGGTCGAGGGACCTAA TA

Avertin preparation

Timing: 2 days

4. Prepare the stock solution (1.6 g/mL).

Add 25 g 2,2,2-tribromoethanol (Avertin) and 15.5 mL tert-amyl alcohol in the dark. Stir on magnetic stirrer until the Avertin is dissolved (approximately 12 h). Avertin stock is light sensitive and hydroscopic and must be used away from light, and can be stored at 4°C for 1 year.

5. Prepare the working solution (20 mg/mL).
   a. Mix 0.5 mL Avertin stock solution and 39.5 mL phosphate-buffered saline (PBS) in a glass vessel. Seal the container with parafilm, wrap in foil to avoid light and stir on a magnetic stirrer for approximately 12 h or until dissolved.
   b. Filter-sterilize the solution through a 0.22-μm filter and store at 4°C. The working solution can be aliquoted into 5-mL aliquots in sterile vials wrapped in foil or in a dark, capped bottle at 4°C for one year

⚠ CRITICAL: Avertin should be stored in the dark!

Anti-PD-1 antibody preparation

Timing: 30min

6. Prepare 1-mL aliquots immediately after receiving the anti-PD-1 antibody. The aliquots can be stored at −80°C for at least 1 year. Thaw the antibody on ice before use; note that the mice should be treated with the same antibody batch.
7. Dilute the antibody to 1 mg/mL with PBS, and administer 200 μg antibody at a time per mouse.

Enzyme preparation

Timing: 1h

8. Reconstitute DNase I and collagenase IV from lyophilized powders according to the manufacturer's instructions.
   a. Reconstitute DNase I with PBS at a final concentration of 10 mg/mL and can be store at −20°C for at least 1 year.
   b. Reconstitute collagenase IV with PBS at a final concentration of 25 mg/mL, prepare 100 μL aliquots to avoid repeated freeze-thaw cycles, aliquots can be stored at −20°C for 1 year.

Preparation of cytokine stimulation reagents

Timing: 2h

9. Prepare the cytokine stimulation reagents, phorbol 12-myristate 13-acetate (PMA), ionomycin, and Golgi inhibitor, at 1000 × concentrations. PMA is an analog of diacylglycerol, which is a key mediator in a variety of intracellular signaling pathways. Ionomycin can activate Ca^{2+}-sensitive kinase to regulate gene expression. Brefeldin A which is the main effector of Golgi inhibitor

can block intracellular protein transport processes. Together, these stimulation reagents lead to cytokine accumulation within the cell.

a. Dissolve PMA in DMSO at a final concentration of 0.5 mg/mL, and prepare 10-μL aliquots to avoid repeated freeze-thaw cycles. The aliquots can be stored at −20°C for 1 year.
b. Dissolve ionomycin in DMSO at a final concentration of 1 mM, prepare 10 μL aliquots. The aliquots can be stored at −20°C for 1 year.
c. Golgi inhibitor (Brefeldin A) obtained from BD Biosciences is used according to the manufacturer's instructions. Prepare 10-μL aliquots. The aliquots can be stored at −20°C for 1 year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse PD-1(Clone: RMP1-14)	Bio X Cell	Cat#BE0146; RRID: AB_10949053
Rat IgG2a isotype control, anti-trinitrophenol (Clone: 2A3)	Bio X Cell	Cat#BE0089; RRID: AB_1107769
Anti-CD8-AF647(Clone: 53-6.7)	BioLegend	Cat#Cat#100724 RRID: AB_389326
Dye-eFluor 506	eBioscience	Cat#65-0866-14
anti-CD45-percp/Cy5.5(Clone 30-F11)	BioLegend	Cat#103132 RRID: AB_893340
Anti-CD3-APC (Clone: 17A2)	BioLegend	Cat# :100235 RRID: AB_2561455
Anti-CD8a-PE/Cy7 (Clone: 53-6.7)	BioLegend	Cat#100722 RRID: AB_312761
Anti-IFNγ-Bv421 (Clone: XMG1.2)	BiLegend	Cat#505830 RRID: AB_2563105
Anti-GZMB-FITC (Clone: GB11)	BioLegend	Cat#515403 RRID: AB_2114575
Chemicals, peptides, and recombinant proteins		
Collagenase IV	Invitrogen	Cat#17104019
DNase I	Roche	Cat#10104159001
PMA	Sigma	Cat#P8139
Ionomycin	PeproTech	Cat#5608212
Golgi inhibitor	BD Biosciences	Cat#554724
Percoll	GE Healthcare	Cat#17-0891-01
2,2,2-Tribromoethanol (Avertin)	Sigma-Aldrich	Cat#T48402
DMEM	Gibco	Cat#11995
RPMI-1640	Gibco	Cat#11875
Phosphate Buffered Saline (PBS)	HyClone	Cat#SH30256
Fetal Bovine Serum (FBS)	HyClone	Cat#SV30087.03
Penicillin-Streptomycin	Gibco	Cat#15140-122
HBSS	Gibco	Cat#14170146
Polyethylenimine (PEI)	Polysciences	Cat#22966
Sucrose	Coolaber	N/A
Paraformaldehyde (PFA)	Servicebio	Cat#G1101
Albumin Bovine (BSA)	BioFroxx	Cat#4240
RIPA Buffer	Beyotime	Cat#P0013B
Protease inhibitor cocktail	Roche	Cat# 4693116001
PMSF	Beyotime	Cat# ST506
Trypsin-EDTA	Gibco	Cat#25200056
ACK lysis buffer	Gibco	Cat#A1049201
OCT	Sakura	Cat#4583
DAPI	Sigma-Aldrich	Cat#D9542
tert-Amyl alcohol	aladdin	Cat#B1714001
Tween-20	Sigma-Aldrich	Cat#P7949
Triton X-100	Sigma-Aldrich	Cat# X100

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
image-iT Image Enhancer	Invitrogen	Cat#I36933
One Step Mouse Genotyping Kit	Vazyme	Cat#PD101-01
qPCR Lentivirus Titration Kit	abm	Cat#LV900
Fixation/permeabilization solution	BD Biosciences	Cat#554722
Perm/Wash Buffer	BD Biosciences	Cat#554723
BCA Protein Assay Kit	Thermo	Cat#23227
4× loading buffer	Bio-Rad	Cat#1610747
Experimental models: cell lines		
Tumor-derived cell line (TDCL)	This paper	N/A
CAG-Loxp-mCherry-Loxp-ZsGreen cell line	This paper	N/A
HEK293T	ATCC	Kenneth Irvine lab
Experimental models: organisms/strains		
Mouse: $Kras^{G12D.LSL}$; $P53^{fl/fl}$; $Zsgreen^{LSL}$	This paper	N/A
Mouse: $Kras^{G12D.LSL}$	The Jackson Laboratory	JAX: 008179
Mouse:$P53^{fl/fl}$	The Jackson Laboratory	JAX: 008462
Mouse: $Zsgreen^{LSL}$	The Jackson Laboratory	JAX: 007906
C57BL/6 mouse	Beijing Vital River Laboratory Animal Technology	N/A
Oligonucleotides		
Forward Primer for K-RasG12D wildtype allele: TGTCTT TCCCCAGCACAGT	The Jackson Laboratory	Primer ID: 22907
Common Primer for K-RasG12D allele: CTGCATAGTACG CTATACCCTGT	The Jackson Laboratory	Primer ID: 22908
Forward primer for K-RasG12DLSL mutant allele: GCAGGTCG AGGGACCTAA TA	The Jackson Laboratory	Primer ID: oIMR9592
Software and algorithms		
Cellsens	https://www.olympus-lifescience.com/en/software/cellsens/	N/A
FlowJo V10	BD Biosciences	N/A
GraphPad prism 7	GraphPad Software	N/A
Recombinant DNA		
Lenti-LucOSCre	Addgene	Addgene #22777
psPAX2	Addgene	Addgene #12260
pMD2.G	Addgene	Addgene #12259
Other		
0.22-μm filter	Millipore	Cat#SLGP033RB
0.45-μm filter	Millipore	Cat#SLHP033RB
70-μm cell strainer	BD Biosciences	Cat#352350
SW-41 ultracentrifuge tube	Beckman Coulter	REF#344059
96-well plate	NEST	Cat#701201
24-well plate	NEST	Cat#702001
10 cm petri dish	NEST	Cat#704004
200-mesh filter	Solarbio	Cat#YA0949
BD Verse Cytometer	BD Biosciences	N/A
Embedding molds	Thermo	Cat#1830
NX 50 Cryostat	Thermo	N/A
Mounting Medium	Beyotime	Cat#P0126
FV3000 confocal system	Olympus	N/A
Trans-Blot Turbo Transfer System	Bio-Rad	N/A
CLx Imaging System	Odyssey	N/A

MATERIALS AND EQUIPMENT

DMEM complete medium		
Reagent	Final concentration	Amount
DMEM	-	500 mL
FBS	10%	56 mL
penicillin-streptomycin	100 U/mL	5.6 mL
The medium can be stored at 4°C for 1 month.		

RPMI 1640 complete medium		
Reagent	Final concentration	Amount
RPMI 1640	-	500 mL
FBS	10%	56 mL
penicillin-streptomycin	100 U/mL	5.6 mL
The medium can be stored at 4°C for 1 month.		

PEI solution		
Reagent	Final concentration	Amount
PEI	1 μg/μL	0.1 g
H_2O	-	100 mL
Prepare 1 mL aliquots, the aliquots can be stored at −20°C for 1 year.		

STEP-BY-STEP METHOD DETAILS

$Kras^{Lox\text{-}STOP\text{-}Lox(LSL)\text{-}G12D}$;$Trp53^{flox/flox}$; $ZsGreen^{flox/flox}$ mouse model

Timing: 8 weeks

This section describes how to induce autochthonous tumors in genetically engineered mouse models (GEMMs).

1. Package lentivirus.
   a. HEK293T cell line is cultured and maintained in 10 cm petri dish using 10 mL DMEM (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS, HyClone) and 100 U/mL penicillin-streptomycin (GIBCO) at 37°C and 5% CO_2 in a humidified Thermo fisher incubator.
   b. Produce lentivirus by cotransfecting 293T cells at 70%–80% confluency with 4 μg Lenti-LucO-SCre(Addgene,#22777), 3 μg psPAX2 (Addgene, # 12260), and 1.5 μg pMD2.G (Addgene, # 12259)(DuPage et al., 2011). 1 μg/μL polyethylenimine (PEI) solution was used as transfection reagent, the transfection process are as follows.
      i. Mix the plasmids (9 μg in total) with 1 mL FBS free DMEM, stand for 5 min at 25°C.
      ii. Add 27 μL (3 times the mass of plasmids) PEI solution to the plasmids-DMEM mixture, and stand for 15 min at 25°C.
      iii. Add the transfection mixture to petri dish dropwise.
   c. Change medium within 18 h post transfection, given that viruses are produced around 18 h. Harvest supernatants containing viral particles 48 h after the medium change by collecting the supernatants in a 15-mL sterile tube. Keep everything on ice at all times.
   d. Pellet nonadherent cells and cell debris by centrifugation at 1600 × *g* at 4°C for 10 min and pass the supernatant through a sterile, 0.45-μM low-protein binding filter(Joshi et al., 2015).

⚠ CRITICAL: To obtain high-quality lentivirus, HEK293T should be at rapid growth stage, and the cells should be transfected at 70%–80% confluency.

2. Concentrate viral particles by ultracentrifugation.
   a. Transfer approximately 10 mL lentiviral supernatant to SW-41 ultracentrifuge tubes containing 1.5 mL of 20% sucrose cushion in PBS (w/v). Perform this step slowly to avoid mixing the layers.
   b. Bring a scale into the biosafety cabinet and balance the tubes in the metal bucket. by adding media with virus particles until the tubes are at no more than 0.01 g apart in weight and are filled until 2 mm from the top (also see Figure 6).
   c. Concentrate lentiviral particles by centrifugation in an SW-41 rotor at 100000 × *g* for 2 h at 4°C (the virions will be in the pellet at the bottom of the tube).
   d. Remove the supernatant carefully!
   e. Resuspend the pellet in 200 μL of 1× HBSS buffer.
   f. Immediately titrate the samples and prepare 50 μL aliquots. The aliquots can be stored at −80°C for at least 1 years.
   g. Clean the biosafety cabinet with 10% bleach and then 70% EtOH. Treat the used pipettes and tips with 70% EtOH from biohazardous contamination.

⚠ CRITICAL: This whole process should be performed on ice to maintain the viability of virus. Concentrated virus should be stored at −80°C in single-use aliquots to avoid freezing and thawing (Troubleshooting 1).

3. Lentivirus titration
   a. Titrate lentivirus using the qPCR Lentivirus Titration Kit following the manufacturer's instructions: https://www.abmgood.com/qpcr-lentivirus-titration-titer-kit-lv900-vin.html
   b. For functional titration, titrate lentiviruses expressing Cre by infecting the *CAG-Loxp-mCherry-Loxp-ZsGreen* cell line (termed the Cre reporter).
      i. Seed 1×10^4 Cre reporter cells with 100 μL DMEM complete medium in a 96-well plate.
      ii. Serially dilute the virus 10-fold and replace the medium with 100 μL diluted virus. Count the number of ZsGreen-positive cell colonies 3 days after the medium change.
      iii. Use the two smallest colony numbers to calculate the titer with the following formula: titer = (smallest colony number × dilution rate + the second smallest colony number × dilution rate) ×100/2 TU/mL.
4. Lung Intratracheal intubation (also see the Methods video S1)
   a. Sedate a $Kras^{Lox\text{-}STOP\text{-}Lox(LSL)\text{-}G12D};Trp53^{flox/flox};ZsGreen^{flox/flox}$ mouse (6–8 weeks of age) by intraperitoneal injection of Avertin (female 0.4 mg/g, male 0.45 mg/g of body weight).
   b. While anesthesia sets in, prepare the catheter for intubation. First, blunt the needle of the catheter by cutting the end with scissors. Then, push the catheter completely over the end of the needle.
   c. Confirm the appropriate level of anesthesia by pedal reflex via firm toe pinching.
   d. Fix the mouse on the intubation platform by hooking its upper incisors over a suture and confirm that the chest is vertical underneath the suture.
   e. Place a fiber optic cable between the front legs to illuminate the chest.
   f. Carefully open the mouth of the mouse and pull out the tongue using disinfected flat forceps. Look for the emission of white light to locate the larynx and visualize the epiglottis and arytenoid cartilages.
   g. Once the opening of the trachea is clearly visible, gently slide the catheter into the trachea. The length of the catheter to be inserted depends on the age and size of the animal, since it should not go below the bifurcation to guarantee an even distribution of lung adenocarcinoma cells within the lung. Quickly remove the needle from the catheter.
   h. The proper placement of the catheter in the trachea is indicated by white light shining through the catheter. To confirm the placement of the catheter in the trachea, attach a 1-mL syringe containing water to the catheter. The water in the syringe will rapidly move up and down in accordance with the breathing.

i. Pipette 50 μL of the suspension containing 10^5–10^6 TU virus per mouse into the center of the catheter hub. The suspension should be aspirated immediately. Subsequently, attach a 1-mL syringe and dispense 300 μL of air to ensure a consistent distribution within the lungs.
j. Gently remove the catheter, remove the mouse from the intubation platform, and place it on a heat pad until it recovers from anesthesia.
k. Ten weeks later, sacrifice the mice and isolate the lungs with adenocarcinoma for histological examinations and Tumor derived cell line (TDCL) establishment.

⚠ CRITICAL: To ensure that tumors are evenly distributed in the lung, viruses should be evenly distributed within the lungs. We recommend dispensing 300 μL of air to the trachea after pipetting the virus. The concentration of virus and the time should be recorded (Troubleshooting 2).

TDCL cell line establishment

Timing: 4 weeks

This section describes how to establish a mouse tumor cell line derived from GEMMs.

5. Culture primary tumor tissue.
   a. Wash fresh lung tumor tissue with PBS containing 1% penicillin and streptomycin and mince the tissue into approximately 1-mm diameter pieces with scissors.
   b. Digest the minced tumor tissue with 1 mL 0.25% trypsin–EDTA for 15 min at 37°C.
   c. Remove cell aggregates and tissue fragments with a 70-μm cell strainer.
   d. Collect the cells by centrifugation for 5 min at 200 × *g*, remove the supernatant, resuspend 10000 cells with 500 μL RPMI-1640 complete medium, seed 500 μL cell suspension in 24 well culture plates.
   e. Maintain the cells in 500 μL RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2.
   f. Refresh the tissue culture medium every three days.
6. Isolate single-cell clones.
   a. Trypsinize subconfluent cell monolayers with 100 μL 0.05% trypsin–EDTA for 2 min at 37°C.
   b. Count the cells, dilute cells to 80 cells per 10 mL and seed 100 μL into 96 wells plates.
   c. Culture the cells for 14 days and pick individual colonies using microscopy.

Mouse subcutaneous allograft injection

Timing: 25 days

This protocol describes how to establish the subcutaneous allograft mouse model and monitor tumor growth *in vivo* using mouse tumor cells derived from GEMMs.

7. Expand the cells.
   a. Seed TDCL cells in 10cm well 24 h before transplantation at approximately 50% confluency in RPMI-1640 complete medium. Incubate the cell cultures at 37°C, 5% CO_2, and approximately 95% relative humidity.
   b. On the next day, harvest the cells using 1 mL of trypsin-EDTA (0.05% in PBS) at 37°C for 5 min per 10-cm plate and, subsequently, resuspend the detached cells with 9 mL of RPMI-1640 complete medium.
   c. Count the cells in a hemocytometer and transfer the number of cells needed for the experiments in a 50-mL conical centrifuge tube.

d. Subsequently, centrifuge the cells for 5 min at 300 × *g*, aspirate the supernatant, and, using a pipette, resuspend the cells at a density of 7.5 × 10^6/mL (for the injection of 1.5 × 10^6 TDCL cells per mouse) in PBS.
e. Keep the cells on ice until transplantation.

8. Perform subcutaneous injection.
   a. Prepare C57BL/6 male mice at the age of 6–8 weeks.
   b. Mix the cell suspension and subcutaneously implant 200 μL of the suspension containing 1.5 × 10^6 cells (the number of cells may vary) in the right flank of each mouse.
   c. Once the tumor volume reaches 50–100 mm^3 (6–8 days), randomly separate the tumor-bearing mice into two groups.

Note: We recommend using mice of the same genetic background and sex as the donor of tumor cells to guarantee the recipient mice tolerant well to the tumor cells.

9. Measure the tumor volume.
   a. Determine the tumor volumes by measuring the length (l) and width (w) with a Vernier caliper every other day and calculating the volume with the formula ($V = 0.5 \times l \times w^2$).
   b. At the end of the experiment (approximately 25 days), the mice are sacrificed, and tumors are isolated by dissection, weighed and used for in vitro experiments.

Note: The mean tumor diameter should not exceed 20 mm according to the tumor burden guideline.

Anti-PD-1 treatment

Timing: 3 weeks

This section describes anti-PD-1 antibody treatment in tumor-bearing mice and how to monitor the tumor volume. We recommend using mice of the same sex and age to reduce individual differences.

10. Intraperitoneally inject mice with 0.2 mg anti-PD-1 antibody(aPD-1) or Isotype control (IgG) 3 days after inoculation and treat every 3 days.
11. Monitor the tumor volume with calipers every 2–3 days to ensure that the tumor volume does not exceed the ethical requirements for animal protection.

Tumor-infiltrating lymphocyte isolation

Timing: 1 day

This step included dissection of the tumor tissue, dissociation of the tumor into single-cell suspension and lymphocyte isolation. We recommend using freshly isolated tumor tissue and that the processing time is minimal to ensure cell viability.

12. Clean and sterilize surgical instruments, filters, and containers.
13. Sacrifice the mice, dissect the tumors and carefully remove the skin. Cut 0.5 g tumor tissue for lymphocyte isolation, transfer the tumor tissue to a 50-mL tube with 1 mL FBS free DMEM and keep on ice till all the tumor tissue is dissected. It often takes 1 h for one person to dissect 10 mice.
14. Gently cut the tumor into approximately 1-mm diameter pieces using scissors (perform this step within 2 min).
15. Prepare the digestion solution
    a. Add 5 mL PBS to sterilized bottle.
    b. Add 50 μL 25 mg/mL Collagenase IV.

c. Add 20 μL 1 mM DNase I.
d. Mix well by shaking

16. Add 5 mL digestion buffer to the 50-mL tube which containing tumor pieces, and incubate the sample in a shaker at 200 rpm for 1 h at 37°C.

⚠ CRITICAL: This step should be performed very gently and quickly. Cell viability will decrease sharply if the procedure is too long. Note that the quality of collagenase is also crucial and determines the quantity and activity of cells.

17. Obtain a single-cell suspension.
    a. Add 15 mL PBS to stop the digestion.
    b. Repeatedly pipette the tissue suspension with a dropper.
    c. Pass the tissue suspension through a 200-mesh filter and then collect the cell suspension.
18. Lyse blood cells.
    a. Precool the centrifuge to 4°C.
    b. Centrifuge the cell suspension at 460 × *g* for 5 min to collect the cell pellet.
    c. Discard the supernatant, resuspend the cells in 1 mL ACK lysis buffer and place on ice for 1 min.
    d. Add 10 mL PBS to stop the lysis and centrifuge the cell suspension at 460 × *g* for 5 min.
19. Prepare 40% (v/v) Percoll gradient solution.
    a. Add 6 mL serum free DMEM to a sterilized bottle.
    b. Add 0.4 mL 10 × PBS.
    c. Add 3.6 mL Percoll.
    d. Mix well by swirling.
20. Discard the supernatant, resuspend the cells in 10 mL 40% (v/v) Percoll gradient solution, and centrifuge the cell suspension at 550 × *g* for 35 min.
21. Carefully remove the supernatant, which contains a large number of tumor cells, with a dropper. The resulting cell pellet is enriched for lymphocytes.
22. Resuspend the pellet in 10 mL PBS and centrifuge at 460 × *g* for 5 min to wash the cells.

Discard the supernatant, resuspend the pellets in 1 mL DMEM containing 3% FBS, and count the living cells by trypan blue staining. Transfer equal numbers of living cells (5 × 10^6 is recommended) to 1.5-mL tubes for further flow cytometry analysis (Troubleshooting 3).

Flow cytometry analysis

Timing: 2 days

Day 1

23. Prepare culture medium with cytokine stimulations reagents.
    a. Add 0.6 mL DMEM with 10% FBS.
    b. Add 0.6 μL 0.5 mg/mL PMA stock.
    c. Add 0.6 μL 1 mM Ionomycin stock.
    d. Add 0.6 μL golgi inhibitor stock.
    e. Mix well by vortexing.
24. Centrifuge the lymphocytes at 460 × *g* for 5 min at 4°C, discard the supernatant and resuspend the cell pellets with 600 μL culture medium containing stimulations reagents.
25. Transfer the cell suspension to a 24-well plate and incubate the cells in 5% CO_2 at 37°C for 3 h.

26. Stain for flow cytometry
    a. Allow lymphocytes to attach to the bottom of the plates, resuspend and transfer the cells to 96-well plates. Centrifuge the 96-well plate at 460 × *g* for 2 min at 4°C and discard the

supernatant. The capacity of a 96 well is 300 μL; therefore, transfer the solution twice to collect all the cells.

b. During centrifugation, prepare FACS buffer, which is 0.2% BSA (w/v) in PBS.

c. Resuspend the cells in 200 μL FACS buffer, centrifuge the plate at 460 × *g* for 2 min at 4°C, and discard the supernatant.

d. Prepare the antibody solution.

Antibodies against Cell membrane markers		
Antibody	Dilution rate	Amount
FACS buffer	N/A	50 μL
Fixable Viability Dye -eFluor 506	1:400	0.125 μL
anti-CD45-percp/Cy5.5	1:200	0.25 μL
anti-CD3-APC	1:200	0.25 μL
anti-CD8a-PE/Cy7	1:200	0.25 μL

e. Resuspend the cell pellets in 50 μL antibody and incubate the cells at 25°C for 15 min. Note that the following steps need to be performed away from light.

f. Centrifuge the plate at 460 × *g* for 2 min and discard the antibody.

g. Resuspend the cell pellets in 200 μL FACS buffer, centrifuge the plate at 460 × *g* for 2 min and discard the supernatant.

h. Resuspend the cells in 150 μL fixation/permeabilization solution and fix the cells at 4°C for 40 min.

Pause point: The fixed cells can be stored at 4°C for 24 h. Wash the cells with 200 μL FACS buffer, centrifuge and resuspend the cells in 200 μL FACS buffer. The next step can be performed on the second day.

Day 2

i. Wash the cells with 200 μL of 1 × perm/wash buffer, centrifuge the plate at 460 × *g* for 2 min, and discard the supernatant.

j. Prepare the antibody solution.

Cytokine antibodies		
Antibody	Dilution rate	Amount
1×Perm/Wash Buffer	N/A	50 μL
anti-IFNγ-Bv421	1:200	0.25 μL
anti-GZMB-FITC	1:200	0.25 μL

k. Resuspend the cell pellets in 50 μL antibody solution and incubate the cells at 4°C for 1 h.

l. Centrifuge the plates at 460 × *g* for 2 min, discard the antibody, and wash the cells with 200 μL FACS buffer.

m. Centrifuge and resuspend the cells in 200 μL FACS buffer. Pass the cell suspension through a 200-mesh filter and analyze with a BD Verse cytometer.

n. Refer to Figure 3 for the gating strategy. We recommend collecting 10,000 CD8+ T cells for further analysis (Troubleshooting 4).

Note: Isotype control is recommended for cytokine staining, it helps you estimate the non-specific binding. For markers that are clearly bimodal, such as CD45 and CD8, there is no need to set up isotype controls.

Immunofluorescence analysis of CD8$^+$ T cells

Timing: 2 days

Day 1

This protocol describes how to process tumor tissue into slices and perform CD8$^+$ T cell immunofluorescence to evaluate CD8$^+$ T cell infiltration per tumor area.

27. Embed the tissue.
    a. Fix the freshly isolated tumor tissues with 1% paraformaldehyde (PFA) at 4°C for 12 h.

⚠ CRITICAL: For CD8 staining, the concentration of PFA is critical, and over 2% will result in a weak CD8 signal (Liu et al., 2015).

    b. Incubate the tissue with PBS (2 h), PBS with 15% sucrose (w/v) (2 h) and PBS with 30% sucrose (8 h) until the tissues are fully dehydrated.
    c. Place the dehydrated tissue in an embedding box, add OCT until the tissue is fully covered and seal the box. Incubate at 4°C for 12 h.
    d. Snap freeze the tissue.
        i. Obtain dry ice and transfer to a foam box; press the embedding box on the dry ice to create a box-shaped groove.
        ii. Place the box with tissue into the groove and freeze for 20 min.
        iii. Store the tissue at −20°C after OCT fully freezes.

28. Obtain tissue sections.
    a. Cut the tissue into 5-μm-thick slices with a cryostat and transfer to a glass slide.
    b. Incubate the slices at 25°C for 2 h to dry.
    c. Store the slices at −20°C.

29. Perform immunostaining of tissue sections.
    a. Add 500 μL PBS to the tissue section, let stand, and wash for 10 min.
    b. Discard the liquid by vacuum, draw lines on both sides of the tissue with a PAP pen, and wash with PBS again.
    c. Place the slices in a humidified box, add a drop of image enhancer (Invitrogen, CAT: I36933), and incubate at 25°C for 30 min.
    d. Rinse the slices with PBS and permeabilize with 0.3% (v/v) Triton X-100 in PBS (PBS-Triton) at 25°C for 30 min.
    e. Prepare the antibody solution; mix well by vortex.

Immunofluorescence antibodies

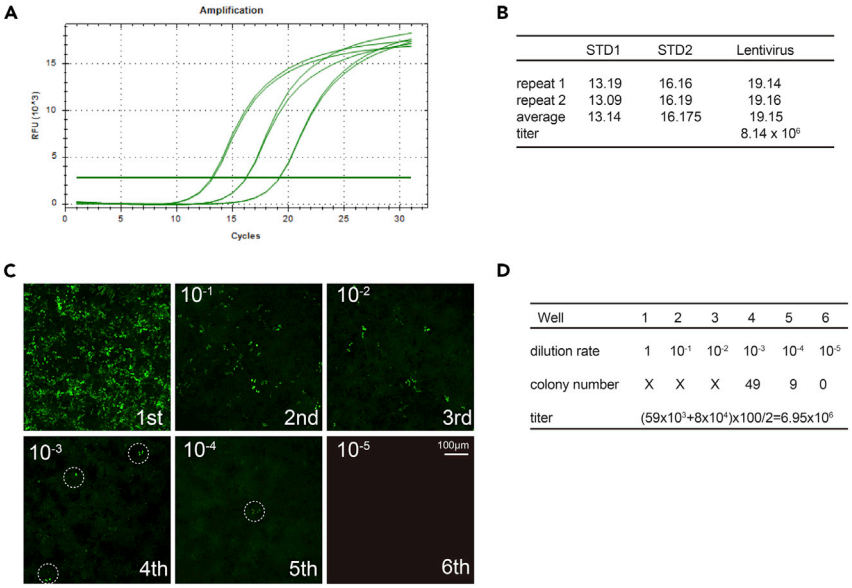
Antibody	Dilution rate	Amount
0.3% PBS-Triton	N/A	100 μL
anti-CD8-Alexa Fluor 647	1:200	0.5 μL
DAPI (10 μg/mL)	1:50	2 μL

    f. Add 100 μL antibody solution to the slice and incubate in a humidified box at 4°C for 12 h

⚠ CRITICAL: Do not perform antigen retrieval as the CD8 signal will be completely lost.

Day 2

    g. Discard the antibody and rinse the slices with PBS-Triton four times.
    h. Add a drop of mounting medium, place the coverslip on the slices and seal with nail polish.



	STD1	STD2	Lentivirus
repeat 1	13.19	16.16	19.14
repeat 2	13.09	16.19	19.16
average	13.14	16.175	19.15
titer			8.14 x 10^6

Well	1	2	3	4	5	6
dilution rate	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
colony number	X	X	X	49	9	0
titer	$(59x10^3+8x10^4)x100/2=6.95x10^6$					

Figure 1. Lentivirus titration method

(A and B). Titrate lentivirus using the qPCR Lentivirus Titration Kit following the manufacturer's instructions. Amplification curve of qPCR (A). Example of titer calculation by Cq value (B).

(C and D). Titrate lentiviruses expressing Cre by infecting the *CAG-Loxp-mCherry-Loxp-ZsGreen* cell line. Representative images of infected cells in indicated well, dotted circle indicate one single colony (C, Scale bar show 100 μm). Functional titer calculation of virus from Figure 1B (D), note that Figure 1C are the representative images to show the cell colonies for counting, all the positive clones in one well should be counted as the colony number.

i. Obtain whole tumor images by stitching images collected with a 10 X objective. Scan the sections by an Olympus FV3000 confocal system with a 60× silicone oil objective to get zoom in images. (Figure 5A). Troubleshooting 5

Western blot analysis

Timing: 2 days

30. Lyse the tumor tissues.
    a. Homogenize and sonicate the tissue in 500 μL RIPA buffer in the presence of 1% (v/v) protease inhibitor cocktail and 2% (v/v) PMSF on ice. The freshly dissected tumor tissue can be stored at −80°C for few months.
    b. Centrifuge at 12,000 × *g* for 15 min at 4°C and collect the supernatant.
31. Determine protein concentrations using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions: https://www.thermofisher.com/order/catalog/product/23227#/23227. Heat the protein at 98°C for 5 min with 4× loading buffer.
32. Separate the sample by SDS-PAGE and transfer to PVDF membranes using the Trans-Blot Turbo Transfer System.
33. Perform immunostaining.
    a. Block the membranes in 5% nonfat milk (Bio-Rad, USA) for 1 h at 25°C .
    b. Incubate the membranes with primary antibodies diluted in TBS-T (0.1% Tween) supplemented with 3% BSA at 4°C for 12 h.
    c. Wash the membranes with TBS-T 4 times.
    d. Incubate the membranes with fluorescent secondary antibodies for 1 h at 25°C.
    e. Wash the membranes and visualize the bands using the Odyssey CLx imaging system.

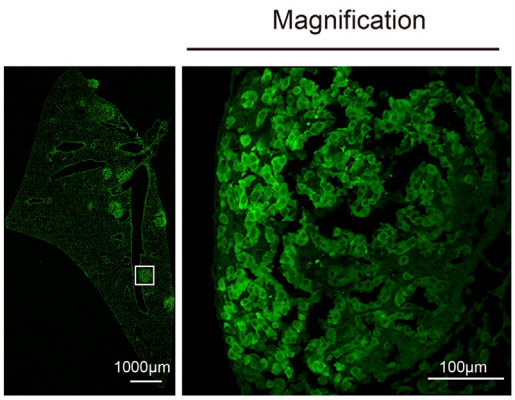


Figure 2. Lung confocal section of KPZ mice at 10 weeks after lentivirus infection

Zsgreen showed tumor mass indicating tumor cells with Zsgreen (green) and the inserts show lower magnifications. Scale bars show 1,000 μm (left) and 100 μm (right). Figure reprinted with permission from Yu et al., 2021.

EXPECTED OUTCOMES

For lentivirus titration, the titer measured by qPCR should be similar to the functional titers (Figure 1).

For KPZ model establishment, the tumor type should be adenocarcinoma according to tumor section (Figure 2).

For lymphocyte isolation, 1×10^7 cells can be obtained from 0.5 g tumor tissue, with over 70% living cells, of which lymphocytes account for approximately 30% (Figure 3).

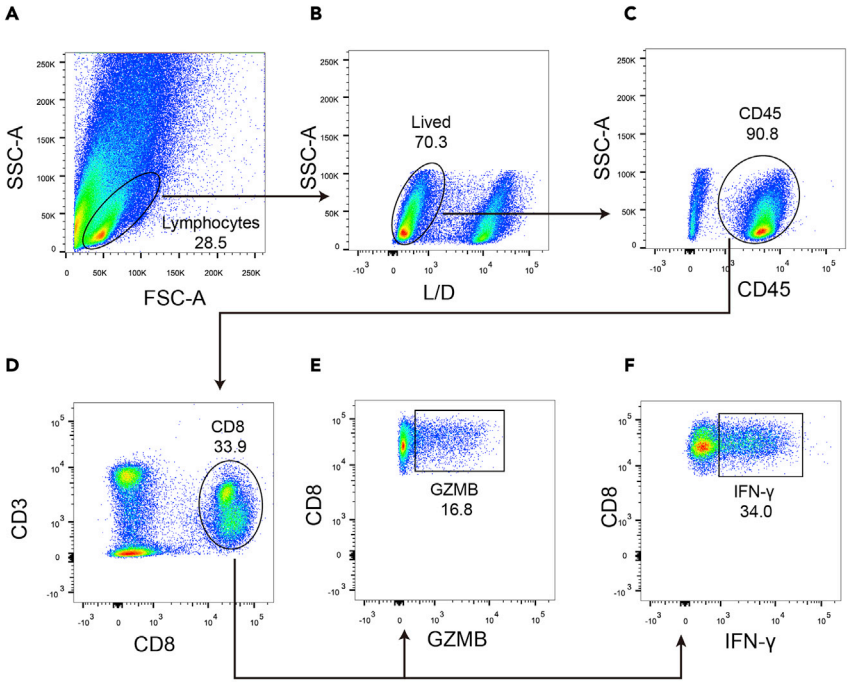


Figure 3. Gating strategy for analysis of cytotoxic T lymphocyte (CTL) activity

Lymphocytes were initially gated by FSA and SSC (A), live gate to exclude viability dye positive dead cells (B), gated on $CD45^+$, $CD3^+$, $CD8^+$ cells successively, $CD8^+$ T cells defined as $CD45^+$, $CD3^+$, $CD8^+$ cells (C and D), the IFN-γ^+ cells and $GZMB^+$ cells in $CD8^+$ T cells were gated respectively (E and F).

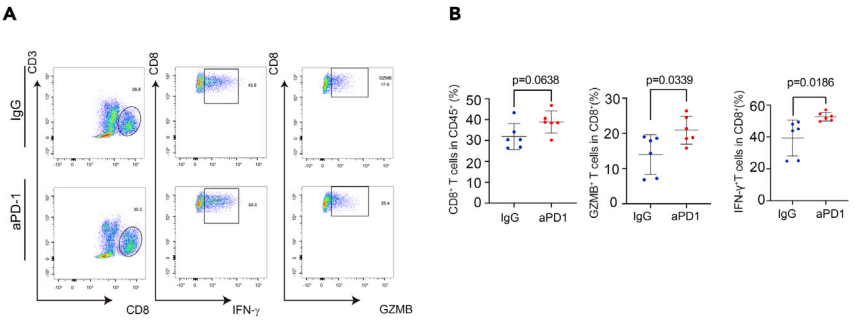


Figure 4. Analysis of CTL activity in tumors that are sensitive to anti-PD-1 therapy by flow cytometry

Representative images of flow cytometry analysis of $CD8^+$, $GZMB^+$, and IFN-γ^+ Cells in TDCL tumors, $CD45^+$ cells were isolated from subcutaneous primary tumor tissues (A), plots showing the mean percentage ± SEM of $CD8^+$ T cells in $CD45^+$ cells, $GZMB^+$ cells, and IFN-γ^+ cells in tumor treated with IgG and aPD-1(n=6 for each group), two-tailed t test (B). Figure reprinted with permission from Yu et al., 2021.

For flow cytometry and immunofluorescence analysis, if the tumor is sensitive to immunotherapy, the frequency of $GZMB^+$ cells in $CD8^+$ T cells, the frequency of IFN-γ^+ cells in $CD8^+$ cells (Figure 4) or $CD8^+$ T cell infiltration (Figure 5B) may be significantly increased, indicating upregulated cytotoxic T lymphocyte activity.

QUANTIFICATION AND STATISTICAL ANALYSIS

For flow cytometry data, the frequency of each population is calculated by FlowJo software. Immunofluorescence images are analyzed using Cellsens software. The value is calculated by calculating the total $CD8^+$ T cell number/whole tumor area.

LIMITATIONS

While these models are of particular value to investigate fundamental processes in lung tumor development, they require extensive mouse breeding, and the experiments are time-consuming.

The tumor size and the number of infiltrating lymphocytes affect the cell yield, and tumors less than 0.2 g is hard to finish the analysis with enough lymphocytes. We recommend treating until the tumor reaches 500 mm^3.

Immunofluorescence of CD8 can partially reflect T cell infiltration, but tumors are highly heterogeneous; thus, analyzing a single slice does not represent the whole tumor. We recommend cutting slices from 3 different layers of the tumor and averaging the numbers.

TROUBLESHOOTING

Problem 1

The lentiviral titer is not high enough (step 2).

Potential solution

There are many factors affecting the titer of the lentivirus, such as the quality of the HEK293T cells and transfection reagent and the purity of the plasmids. Therefore, the cells should be in good condition, the transfection reagent should be fresh and free of quality problems, the plasmid should be highly purified, and the sequence should be verified before transfection.

Problem 2

The *KP* mice do not form tumors after virus intubation (step 4).

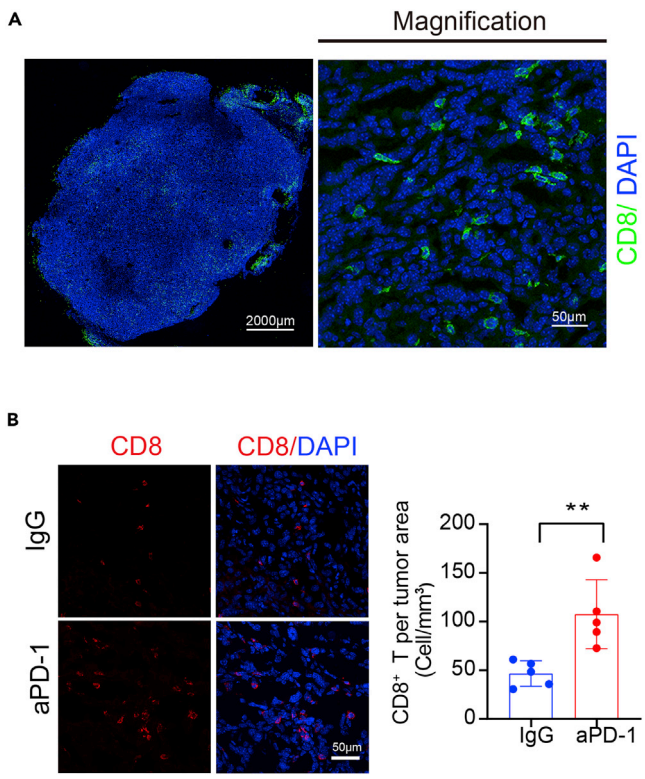


Figure 5. Analysis of CD8$^+$ T cell infiltration by immunofluorescence

(A) Frozen sections of tumor tissue were stained for CD8 (green) and DAPI (blue). The whole tumor images were acquired by stitching images obtained using a 10 X objective (left panel), and the higher magnification images were scanned by a 60 X objective (right panel). Scale bars show 2,000 μm (left) and 50 μm (right).

(B) Analysis of CD8+ T cell infiltration of tumors that are sensitive to anti-PD-1 therapy, the values are calculated by counting total CD8$^+$ T cell number/whole tumor area. n=5 for each group. Scale bar shows 50 μm. Data are mean ± SEM, two tailed T test. Figure reprinted with permission from Yu et al., 2021.

Potential solution

First, the genotype of the experimental mice should be verified before lung intratracheal intubation. Second, it is important to ensure that the virus is delivered to the trachea but not to the esophagus. To confirm the placement of the catheter in the trachea, a 1-mL syringe containing water is attached to the catheter. The water in the syringe will rapidly move up and down in accordance with the breathing. Finally, the titer and viability of the virus you used should not be too low, functional titer over 10^6 TU/mL is recommended.

Problem 3

The isolated lymphocytes are not enough, and the ratio of living cells is low (step 22).

Potential solution

Ensure that all the processes are carried out on ice, the tumor tissue is stored in precooled DMEM while waiting, and the procedure is as short as possible. When cutting up the tissues, ensure that the tissue pieces are not too small. Use collagenase IV with high quality and prepare aliquots to avoid freeze-thaw cycles. The activity of collagenase is important.

Problem 4

Undesirable signal in FC (step 26).

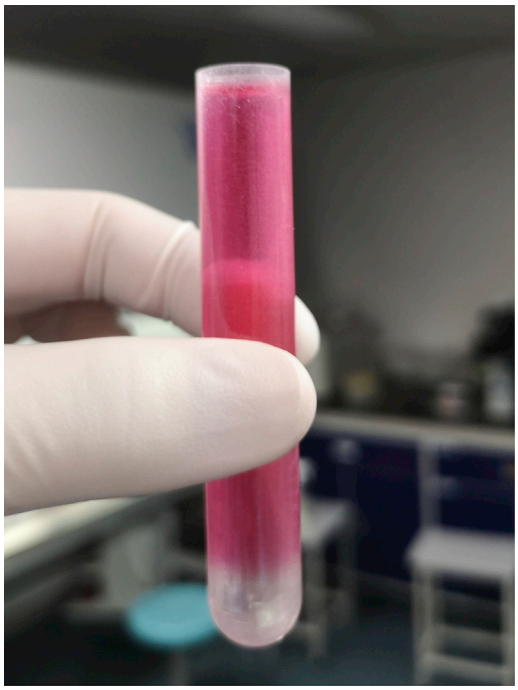


Figure 6. Lentivirus-sucrose cocktail in ultracentrifuge tube
Related to step 2b.

Potential solution

Proper controls can be set up for trouble shooting. First, isotype control can be used to detect non-specific binding, appropriate blocking step can be added before staining if the isotype group showing high background (e.g., FC-receptor blocking). In addition, optimize the amount of antibody used by titration assay can further reduce nonspecific binding. Second, using fluorescence-minus-one control (FMO) can provide information about the potential compensation problem.

Problem 5

No CD8 T cell signal in IF (step 29).

Potential solution

CD8 antigen is sensitive to PFA fixation and antigen retrieval process. Fix the tissue with 1% PFA within 12 h is critical to achieve bright CD8 signal. In addition, we have also tested the CD4 staining condition, CD4 stained well in tissues fixed with 4% PFA for 24 h.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shuguo Sun (Shuguo@hust.edu.cn).

Materials availability

This protocol does not generate new materials.

Data and code availability

This paper did not generate any new datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100595.

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AUTHOR CONTRIBUTIONS

M.Y., J.L., X.Z., and M.S. contributed to the establishment of KPZ model, TDCL cell line, allograft model, and WB analysis; Z.P., T.D., and S.L. contributed to anti-PD-1 treatment, TILs isolation and FC, and IF analysis; T.D., J.L, and W.G. contributed to mice genetics; S.S., M.Y., and Z.P., contributed to experimental design, data analysis, and manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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