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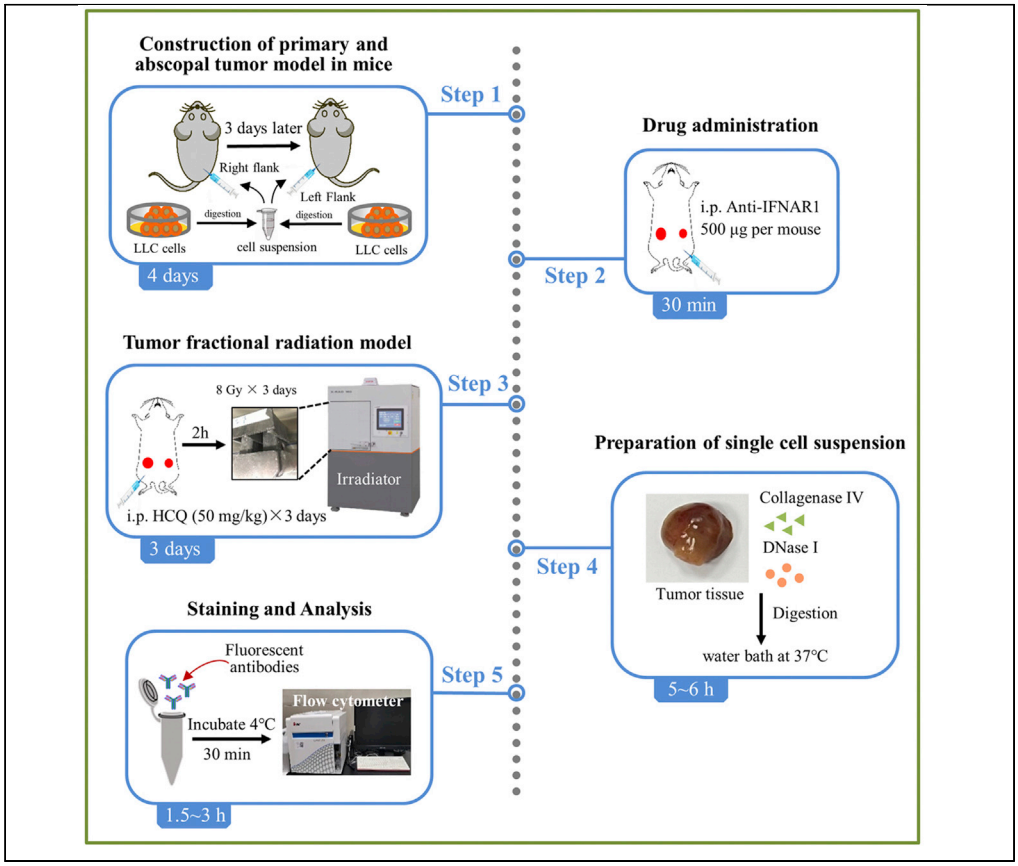
Immunostaining of tumor-infiltrating immune-cell-related markers and cytokines in the tumor fractional radiation model of host PD-L1-deficient mice



In this protocol, we describe how to explore immune function in radiation-induced anti-tumor abscopal effect (ATAE) in PD-L1-deficient (PD-L1$^{-/-}$) mice. We detail steps for primary and abscopal tumor model establishment in mice, tumor fractional irradiation scheme, and isolation of single cells from tumor tissues. Furthermore, we describe the analysis of the proportion of immune cells infiltrated in tumors and the specific staining of cytokines released from immune cells to confirm the ability of CD8+ T cells in killing tumors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Xinrui Zhao, Chunlin Shao

clshao@shmu.edu.cn

Highlights

Protocol to construct mouse xenograft tumor model for studying the ATAE

Hydroxychloroquine sulfate administration to mice by intraperitoneal injection

Detailed steps for single-cell isolation from tumor tissue by enzymatic digestion

Flow cytometry analyses of staining immune cells and cytokines

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Immunostaining of tumor-infiltrating immune-cell-related markers and cytokines in the tumor fractional radiation model of host PD-L1-deficient mice

Xinrui Zhao[1,2] and Chunlin Shao[1,3,*]

[1]Institute of Radiation Medicine, Shanghai Medical College, Fudan University, Shanghai 200032, China

[2]Technical contact: 19111140003@fudan.edu.cn

[3]Lead contact

*Correspondence: clshao@shmu.edu.cn
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SUMMARY

In this protocol, we describe how to explore immune function in radiation-induced anti-tumor abscopal effect (ATAE) in PD-L1-deficient (PD-L1$^{-/-}$) mice. We detail steps for primary and abscopal tumor model establishment in mice, tumor fractional irradiation scheme, and isolation of single cells from tumor tissues. Furthermore, we describe the analysis of the proportion of immune cells infiltrated in tumors and the specific staining of cytokines released from immune cells to confirm the ability of CD8+ T cells in killing tumors.

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

BEFORE YOU BEGIN

Radiotherapy is conventionally applied in antagonizing primary tumors and metastases.[2,3] Currently, the combination of radiotherapy with immunotherapy is increasingly attracted the attentions in the study of radiation-induced anti-tumor abscopal effect (ATAE).[4,5] Exploring the change of tumor microenvironment and the crucial functions of immune cells in the processes of anti-tumor is of great significance to improve the efficacy of radioimmunotherapy (RIT). This protocol gives a step-by-step guidance for the construction of xenograft tumor model and fractional irradiation model, the isolation of single cells from tumor tissues to detect the ratio of immune cells infiltrated in the tumors, and the measurement of cytokines released from CD8+ T cells.

Institutional permission

All animal experiments were approved by the Animal Welfare and Ethics Committee of Fudan University.

Culture of Lewis lung carcinoma (LLC) cells

Timing: 1 week

1. Thaw of frozen LLC cells.
   a. Take out the frozen LLC cells from the liquid nitrogen tank.
   b. Place them in a 37°C water bath immediately, and shake them quickly to accelerate the melting of cells.
   c. Put the melted cryopreservation tube into the centrifuge immediately.
   d. Centrifuge at 100 g for 4 min.

e. Discard the supernatant,
f. Add 1 mL of complete DMEM medium (supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin) to resuspend the cells.
g. Transfer the cell suspension to a 10-cm culture dish supplemented with 7 mL medium.
h. Place cells in an incubator for further culture under 5% CO_2 at 37°C.

2. Cell passage.
   a. Observe LLC cells under an inverted microscope. When the cell density reaches 80%–90% confluence, discard the culture medium and wash the cell with PBS gently for 2–3 times.
   b. Discard PBS buffer.
   c. Add 1 mL trypsin along the dish wall slowly, shake the dish gently and evenly, place the dish in a 37°C incubator for about 1 min.
   d. Add 1 mL complete medium to terminate the digestion when the cells shrink and become round observed under a microscope.
   e. Dissociate the cells with plastic pipette gently.
   f. Collect the cells into a 15 mL centrifuge tube.
   g. Centrifuge at 100 g for 5 min.
   h. Discard the supernatant.
   i. Add 1 mL complete medium to resuspend cells.
   j. Take about one-third of the cells into a 10-cm dish containing 10 mL medium, and shake the dish.
   k. Put the cells in a 5% CO_2 cell incubator for further culture at 37°C.

Note: When the cells grow to nearly 100% confluence in the 10-cm culture dish, the number of cells is about 1×10^7 cells.

Genotyping of mice

Timing: 5–6 h

Male PD-L1 wide type (wt) and PD-L1$^{-/-}$ C57BL/6J mice (7–8 weeks) purchased from Shanghai Model Organisms Center (Shanghai, China) were used in this protocol. The offsprings of mice are genotyped before each experiment. The experimental procedures are shown in Figure 1A.

3. Label each experimental mouse with ear-tag.
   a. Grasp the skin of the mouse neck with one hand.
   b. Pass the labeled ear-tag through the middle of one ear of the mouse quickly and accurately with an ear mark plier.

Note: Put the ear tag plier as close to the root of the mouse ear as possible to avoid the mouse scratching the ear-tag off.

4. Hold the mouse tightly with one hand, and cut off the tail end (0.5 cm in length) with scissors, and put it into a 1.5 mL centrifuge tube on ice.
5. Mouse tail tissues were lysed using Mouse Direct PCR Kit (containing Buffer L and Protease Plus) to extract genomic DNA. Add 100 μL lysate (Buffer L: Protease Plus=50: 1) to each tube.
6. Put the sample tube in a water bath and lysed the tissue at 55°C for 30 min.
7. Put the EP tube in the metal bath at 95°C for 5 min to inactivate the protease.
8. Centrifuge the sample at 25°C at 300 g for 5 min and transfer the supernatant containing genomic DNA to a new EP tube.
9. Store the supernatant at −20°C immediately, or conduct PCR assay as below.
10. Mix the genomic DNA sample with primers for PCR assay.
    a. Set up the PCR reaction system.

Reagent	Amount
DNA template	1 μL
2× M-PCR OPTI™ Mix (Dye Plus)	10 μL
10 μM PD-L1 primer 1	0.5 μL
10 μM PD-L1 primer 2	0.5 μL
ddH_2O	8 μL
Total	20 μL

b. Run the PCR instrument.

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15 s	35
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

c. Set up the PCR reaction system.

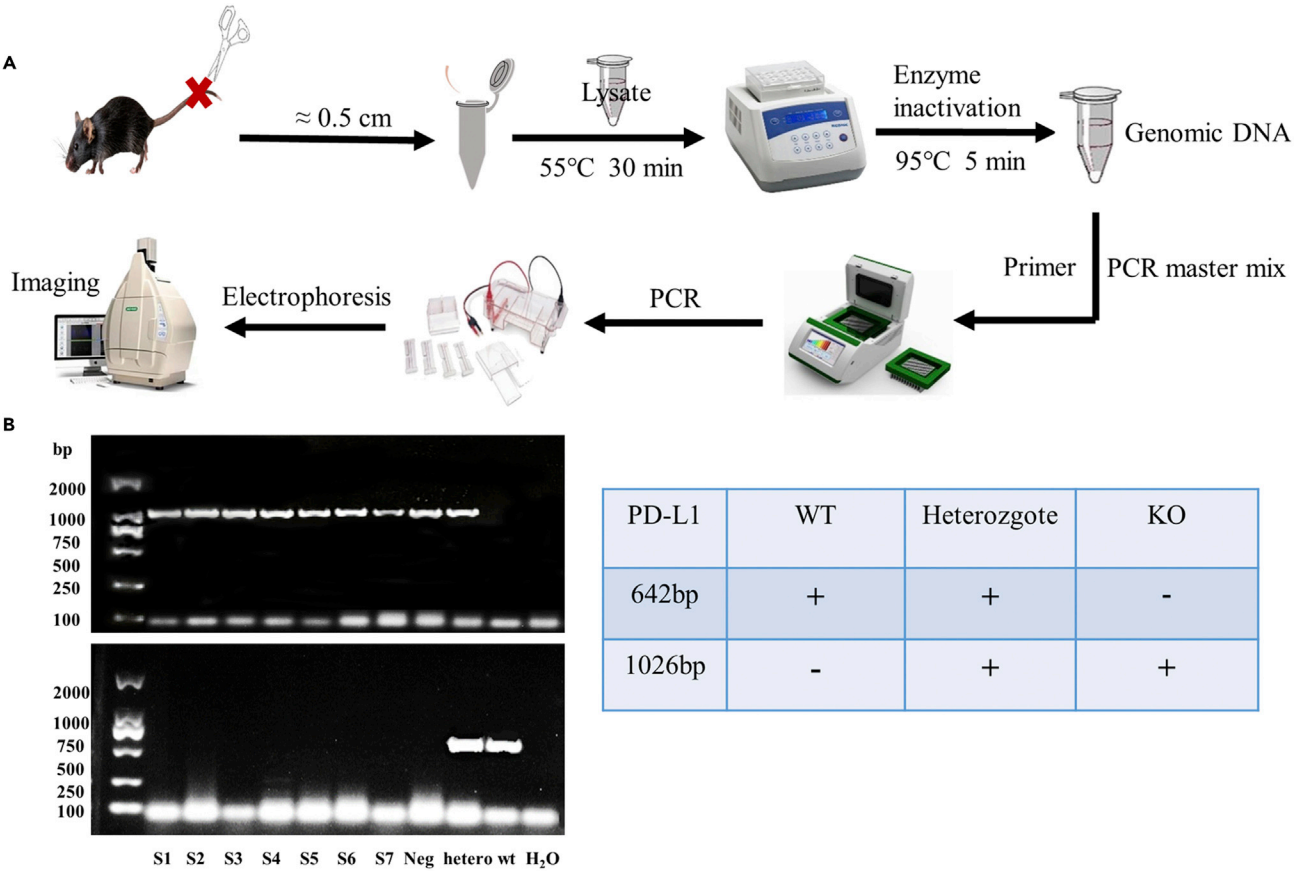
Reagent	Amount
DNA template	1μL
2× M-PCR OPTI™ Mix (Dye Plus)	10 μL
10 μM PD-L1 primer 3	0.5 μL
10 μM PD-L1 primer 4	0.5 μL
ddH_2O	8 μL
Total	20 μL

d. Run the PCR instrument.

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15 s	35
Annealing	60°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

11. Dissolve agarose powder in TAE buffer to prepare 1.5% agarose gel (add 0.01% gel containing YeaRed Nucleic Acid Gel Stain),
12. Run agarose gel electrophoresis at a constant pressure of 100 v/cm for 50 min.
13. Observe the band positions with a gel imager. The relationship between the band positions and PD-L1 genotypes is shown below.
    a. PD-L1 wild type (PD-L1$^{+/+}$): only 642 bp band.
    b. PD-L1 heterozygote (PD-L1 $^{+/-}$): both 642 bp and 1,026 bp bands.
    c. PD-L1 homozygote (PD-L1$^{-/-}$): only 1,026 bp band.

The characteristic DNA band images of different PD-L1 genotypes are shown in Figure 1B.



PD-L1	WT	Heterozgote	KO
642bp	+	+	-
1026bp	-	+	+

Figure 1. Schematic diagram of the identification of mouse genotype

(A) The mouse genotypes can be identified by PCR assay of its genomic DNA extracted from tail tissue.

(B) Typical DNA electrophoretic bands of the tail tissue of mice with different PD-L1 genotypes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC anti-mouse CD3ε (1:250)	Biolegend	Cat # 100306, RRID: AB_312671
PE anti-mouse CD4 (1:200)	Biolegend	Cat # 100408, RRID: AB_312693
APC anti-mouse CD8a (1:200)	Biolegend	Cat # 100712, RRID: AB_312751
PE/Cyanine7 anti-mouse NK-1.1 (1:200)	Biolegend	Cat # 108713, RRID: AB_389363
PE anti-mouse CD279 (PD-1) (1:200)	Biolegend	Cat # 135205, RRID: AB_1877232
PE/Cyanine7 anti-mouse/human CD44 (1:200)	Biolegend	Cat # 103029, RRID: AB_830786
APC/Cyanine7 anti-mouse CD62L (1:200)	Biolegend	Cat # 104427, RRID: AB_830798
PE/Cyanine7 anti-mouse IFN-γ (1:100)	Biolegend	Cat # 505825, RRID: AB_1595591
Purified anti-mouse IFNAR-1	Biolegend	Cat # 127322, RRID: AB_11149116
TruStain FcX™ PLUS (anti-mouse CD16/32) (1:200)	Biolegend	Cat # 156603, RRID: AB_2783137
Chemicals, peptides, and recombinant proteins		
Hydroxychloroquine sulfate (HCQ)	MedChemExpress	Cat # HY-B1370
Pentobarbital sodium	Sigma	Cat # 11715
Collagenase IV	YEASEN	Cat # 40510ES60
Deoxyribonuclease I (DNase I)	YEASEN	Cat # 10607ES15

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse Direct PCR Kit	Bimake	Cat # B40013
Agarose	Beyotime	Cat # ST004Q
YeaRed Nucleic Acid Gel Stain	YEASEN	Cat # 10202ES76
Fixation Buffer	Biolegend	Cat # 420801
Intracellular Staining Permeabilization Wash Buffer	Biolegend	Cat # 421002
Cell Stimulation Cocktail	Invitrogen	Cat # 00-4975-93
2× M-PCR OPTI™ Mix (Dye Plus)	Bimake	Cat # B45012
Dulbecco's Modified Eagle Medium	Gibco	Cat # 11965118
RPMI 1640	Gibco	Cat # 11875176
Fetal bovine serum	Gibco	Cat # 16000-044
Trypsin	Gibco	Cat # 25200072
70 μm cell strainer	SPL life science	Cat # SPL-93070
Experimental models: Cell lines		
LLC cell line	American Type Culture Collection	Cat # CRL-1642, RRID: CVCL_4358
Experimental models: Organisms/strains		
Mouse: C57BL/6J PD-L1$^{-/-}$ (6–8 weeks, Male)	Shanghai Model Organisms Center	N/A
Oligonucleotides		
PD-L1 primer 1	N/A	AGCTTTGGGTGTGGTTTAGTAGTA
PD-L1 primer 2	N/A	GGCTCTCCCCCTGAAGTTG
PD-L1 primer 3	N/A	GCTACTGCACCTGGCTTCTACCTG
PD-L1 primer 4	N/A	TACCTATCCCTGCACCATTGACCT
Software and algorithms		
GraphPad Prism 8	GraphPad	N/A
FlowJo	Treestar	RRID: SCR_008520

MATERIALS AND EQUIPMENT

1 × PBS buffer

Reagent	Amount	Final concentration
10 × PBS	100 mL	1×
RNase-free water	900 mL	N/A

Store at 4°C for up to 1 year.

50 × Tris acetate electrophoresis buffer (TAE)

Reagent	Amount	Final concentration
Tris base	242 g	2 M
Acetic acid (glacial)	57.1 mL	1 M
0.5 M EDTA pH 8.0	100 mL	50 mM
RNase-free water	To 1,000 mL	N/A

Store at 25°C for up to 6 months.

1 × TAE

Reagent	Amount	Final concentration
50 × TAE	20 mL	1×
RNase-free water	980 mL	N/A

Store at 25°C for up to 6 months.

FACS buffer (500 mL)		
Reagent	Amount	Final concentration
1×PBS	490 mL	N/A
FBS	10 mL	2%
0.5 M EDTA solution (PH=8)	200 μL	2 mM
Store at 4°C for up to 6 months.		

STEP-BY-STEP METHOD DETAILS

Construction of primary and abscopal tumor model in mice

Timing: 4 days

1. Cut off the hair of the left and right flanks of mice to expose the skin.
2. Preparation of cell suspension.
   a. Take the LLC cells seeded in 10-cm dishes from the cell incubator to the biosafety cabinet gently and discard the culture medium.
   b. Add 1 mL PBS buffer and wash for 1–2 times gently.
   c. Discard the PBS buffer.
   d. Add 1 mL trypsin and put it in cell incubator for 1 min to digest cells.
   e. Add 1 mL complete DMEM medium to stop the process of digestion when the cells shrink and become round observed under a microscope.
   f. Collect the dissociated cells into 15 mL centrifuge tubes.
   g. Centrifuge at 100 g for 5 min.
   h. Discard the supernatant.
   i. Add 1 mL PBS buffer to resuspend the cells and take out 100 μL cell suspension to count cells with cell counter.
   j. Adjust the cell concentration to 2×10^7 cells/mL with PBS buffer in a 5 mL centrifuge tube.
   k. Put the tube on the ice immediately.

   Note: Enough cells need to be prepared in advance according to the requirement of tumorigenesis.

3. Mix the cells evenly.
   a. Take 100 μL of the cell suspension with 1 mL syringe,
   b. Inject this cell suspension into the right flank of the mouse subcutaneously.

   Note: make sure that the amount of cells injected into each mouse is 2×10^6 cells.

   c. Label the formed xenograft as the primary tumor. This day is recorded as day 0.

   Note: The cells should be injected subcutaneously within 30 min after suspension.

   ⚠ CRITICAL: The number of tumor cells inoculated to mice should be adjusted according to the immune character of mice and the type of tumor cell line. If the tumorigenicity of cells is poor, the number of tumor cells can be appropriately increased, but not more than 1×10^7 cells/mouse. The number of mice in each group can also be increased to exclude individual differences.

4. On the day 3,[6] prepare LLC cells with a concentration of 2×10^7 cells/mL as the step 2.
5. Inject 100 μL cell suspension subcutaneously into the left flank of above mouse to generate the abscopal xenograft tumor.

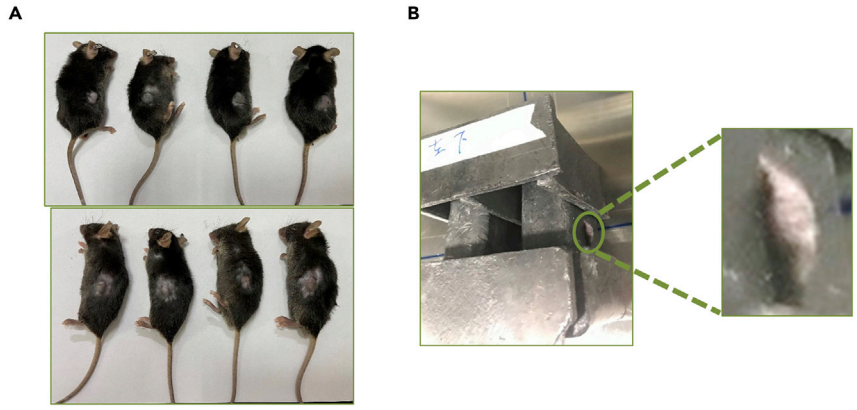


Figure 2. Schematic diagram of local irradiation of tumor in mice

(A) The images of xenograft tumors on both flanks of mice on the day of irradiation.

(B) Tumor irradiation mode. The xenograft tumor is gently clamp out for irradiation, and the rest of mouse body is shielded by lead plates with a total thickness greater than 1 cm.

6. After the completion of tumor inoculation, the mice are fed normally, and the tumor volumes on both flanks are recorded every two days with a digital caliper until the mice are killed.

   Note: For tumor measurements, the tumor volume is calculated with a formula V = (width^2×‴ length)/2.

Drug administration

Timing: 30 min

7. When the primary tumor volume reaches to about 50 mm^3, the PD-L1 wt and PD-L1$^{-/-}$ mice are divided into 7 groups including control, anti-IFNAR-1, HCQ, IR, IR+ anti-IFNAR-1, IR+ HCQ, and IR+ HCQ+ anti-IFNAR-1. Six mice each group.

   Note: In this experiment, the mice were grouped on the day 9 after the injection of primary tumor.

8. Label the mice genotypes, mice number, and treatment methods on an information card of each cage.
9. One day before irradiation, administer anti-IFNAR-1 (500 μg per mouse)[7] into mice by intraperitoneal injection.

   ⚠ CRITICAL: The anti-IFNAR-1 should be administered quickly to ensure the valence of antibody, and the antibody should be injected within 30 min.

Tumor fractional irradiation and drug administration model

Timing: 3 days

On the day 10 after cell injection, the primary tumor with a volume of about 50 mm^3 (Figure 2A) is irradiated with a total dose of 24 Gy X-rays in 3 fractions in 3 consecutive days (8 Gy × 3).

10. Calculate the average weight of mice administrated with HCQ solution.

Note: The experimental mice are weighed and M (g) is set as the mass of each mouse, the number of mice requiring HCQ administration is represented as n, the value of average weight is recorded as $\overline{M}$, $\overline{M} = \frac{\sum_{i=0}^{n} M}{n}$ (g).

11. Calculate the amount of the corresponding reagent.

Note: The dosage of hydroxychloroquine sulfate (HCQ) in this protocol is 50 mg/kg and the required total mass of HCQ powder (m) is calculated as the formula, $m = 0.05\ n\overline{M}\ (mg)$, 100 μL HCQ solution needs to be given at the weight of mice is $\overline{M}$, hence, the total mass of HCQ powder needs to be dissolved in 100 × n μL saline.

12. Weigh the calculated mass of HCQ powder with electronic balance.
13. Dissolve the HCQ powder with saline in the 5 mL EP tube.

Note: Prepare the HCQ solution fresh and used it after fully dissolution.

14. Calculate the volume of HCQ solution injected into each mouse.

Note: The volume of HCQ solution injected into each mouse is calculated by using the formula, $V_n = \frac{M}{\overline{M}} \times 100\ (\mu L)$, and record the value of HCQ solution injected into each mouse. The other mice are given saline as control.

15. Suction off HCQ solution or saline with 1 mL syringe.
16. Inject 100 μL HCQ solution or saline intraperitoneally into mice.
17. Irradiate mice with X ray two hours after drug administration.
    a. Inject 1% pentobarbital sodium intraperitoneally into mice with a dose of 50 mg/100 g for anesthesia.
    b. Put the mouse into a lead mold after the anesthetic takes effect (about 5–10 min).
    c. Clamp out the tumor with tweezers gently, and shield the rest with lead plates (the thickness of lead plate is greater than 1 cm), as shown in Figure 2B.
    d. Irradiate the tumor of mice with 8 Gy X ray.
18. Observe the state of mice closely after irradiation, until all mice wake up.
19. Carry out the same administration of HCQ and irradiation protocols as above in the next two days.

Note: During these three days, to keep the same time point of daily administration, the HCQ administration are all conducted 2 h before irradiation.

Preparation of single cell suspension from tumor tissue

Timing: 5–6 h

20. Kill the mice and strip out the transplanted tumor after 21 days of the first tumor cell injection,
    a. Sacrifice the mice by neck breaking as quick as possible to alleviate the pain of mice.
    b. Pin down mouse on its back using needles.
    c. Spray the mouse body with 75% ethanol.
    d. Cut the mouse skin from the area near the bottom of abdomen to neck with scissors.
    e. Strip out the tumor capsule with a tweezer and dissect out the tumor gently.
    f. Clean the tumor and put it into the marked 50 mL conical tube containing PBS buffer.

Note: All the surgical tools should be autoclaved before use. 50 mL centrifuge tubes have been marked in advance according to the different treatment methods of mice and tumor tissue should be immersed in PBS buffer.

21. Weigh tumor, then cut it into small pieces less than 1 mm and transfer the tumor tissues into a 50 mL conical tube.
22. Add RPMI medium supplemented with 2% FBS to the 50 mL conical tube, 1 mL medium per 1 mg tissue.
23. Add an appropriate amount of collagenase IV into the 50 mL conical tube to a final concentration of 1 mg/mL.
24. Put the conical tube into a shaking bath at 37°C until the tissue is digested.

Note: The time required varies with the type of tumor, but should not exceed 1.5 h to limit the cell viability.

25. Pass the tissue homogenate through a 70 μm cell strainer into a new 50 mL conical tube, and clean the strainer several times with serum-free RPMI.
26. Add DNase into the homogenate and incubate at 37°C for 15 min if the tissue homogenate appears to be caked.

Note: The final concentration of DNase is 10 μg /mL.

27. Collect the cells from tissue homogenate after centrifugation at 400 g for 5 min and resuspend with PBS buffer.
28. Take a small portion of cells for counting and add PBS buffer to adjust the cell density to 1×10^7 cells/mL.
29. Preparation of cells for immunostaining.
    a. For the staining of cell surface antigen, add 100 μL of cell suspension to 1.5 mL EP tubes, 10^6 cells per tube.
    b. For the staining of intracellular cytokine, the 2×10^6 cells are resuspended with 500 μL RPMI medium supplemented with 10% FBS, add 1 μL cell stimulation cocktail (containing protein transport inhibitors) in the cell suspension.
    c. Plate the cell suspension in a 12-well plate and culture cells in an incubator containing 5% CO_2 at 37°C for 16 h.

⚠ CRITICAL: For the cells that need to be used for subsequent culture, the process of preparing single cells from tissues need to be carried out in a biosafety cabinet.

Cell surface antigen staining

Timing: about 1 h

30. Resuspend cells with 100 μL FACS buffer.
31. Pre-incubate with Fc receptor blocker (TruStain FcX™ PLUS) for 5–10 min on ice, 0.25 μg/10^6 cells in 100 μL FACS buffer.
32. Add fluorescence-labeled antibodies to stain the cells and keep away from light. The reference antibody concentrations are: Anti-CD3 (2 μg/mL), anti-CD4 (1 μg/mL), anti-CD8 (1 μg/mL), anti-NK1.1 (1 μg/mL), anti-CD44 (1 μg/mL), anti-CD62L (1 μg/mL), and anti-PD-1 (1 μg/mL).
    a. For the samples of the experimental group, due to the overlapping fluorescence labeling and wavelength of antibodies, the mixing scheme of antibodies is divided into the following two groups:
        i. Anti-CD3, anti-CD4, anti-CD8, anti-NK1.1.
        ii. Anti-CD3, anti-CD8, anti-CD44, anti-CD62L, anti-PD-1.
    b. For the samples of single staining group, we add the sample cells of specific group to 7 EP tubes, respectively, 50 μL cell suspension per tube. Add one antibody (anti-CD3, anti-CD4, anti-CD8, anti-NK1.1, anti-CD44, anti-CD62L, or anti-PD-1) to each EP tube, and the amount of antibody is half of the amount of corresponding antibody in the experimental group.

Note: The whole process of staining should be kept away from light to avoid the loss of fluorescence.

⚠ CRITICAL: Antibodies with intersecting fluorescence wavelengths can't be used to stain cells in the same tube. Each antibody needs to be set with a single staining tube to adjust the fluorescence compensation. In the single staining group, the specific group refers to the experimental group with the highest antigen expression inferred from the pre-experiment, which can ensure that there is a fluorescence signal during fluorescence modulation compensation.

33. Stain the sample at 4°C for 30 min in the dark.
34. Centrifuge at 400 g for 5 min to remove the supernatant.
35. Add 1 mL FACS buffer to wash cells, centrifuge at 400 g for 5 min to remove the supernatant.
36. Add 400 μL FACS buffer to resuspend cells in preparation for flow cytometry analysis.

Intracellular cytokine staining

Timing: 2–3 h

37. Collect the cells and centrifuge at 400 g for 5 min to remove the supernatant after 16 h of cell stimulation.
38. Add 500 μL FACS buffer to wash cells, centrifuge at 400 g for 5 min to remove the supernatant.
39. Resuspend cells with 100 μL FACS buffer.
40. Pre-incubate with Fc receptor blocker (TruStain FcX™ PLUS) for 5–10 min on ice, 0.25 μg/10^6 cells in 100 μL FACS buffer.
41. Add CD8 fluorescent antibody (1 μg/mL) to stain cells in dark condition for 30 min at 4°C. This step is the same as that of staining cell surface antigen.

Note: The whole process of staining should be kept away from light to avoid the loss of fluorescence.

⚠ CRITICAL: If the cells are not sorted, cells need to be stained with antibodies of cell surface marker in advance.

42. Add 1 mL FACS buffer to each tube to wash the cells, and centrifuge at 400 g for 5 min to remove the supernatant.
43. Add 100 μL fixation buffer to fix the cells at 4°C for 20 min.

Note: The purpose of this step is to stabilize the binding of antibodies and antigens on the surface of cell membrane.

44. Centrifuge at 400 g for 5 min to remove the supernatant.
45. Resuspend cells with 100 μL 1× perm/wash buffer.

Note: The main function of perm buffer is to break the cell membrane and clean cells.

46. Add fluorescence-labeled antibody to stain the cell cytokines in dark condition. The reference antibody concentration is 2 μg/mL for IFN-γ.
47. After adding the antibody, stain the sample at 4°C for 30 min in the dark.
48. Add 500 μL 1× perm/wash buffer to wash cells, centrifuge at 400 g for 5 min to remove the supernatant.
49. Add 400 μL FACS buffer into the cells for flow cytometry analysis.

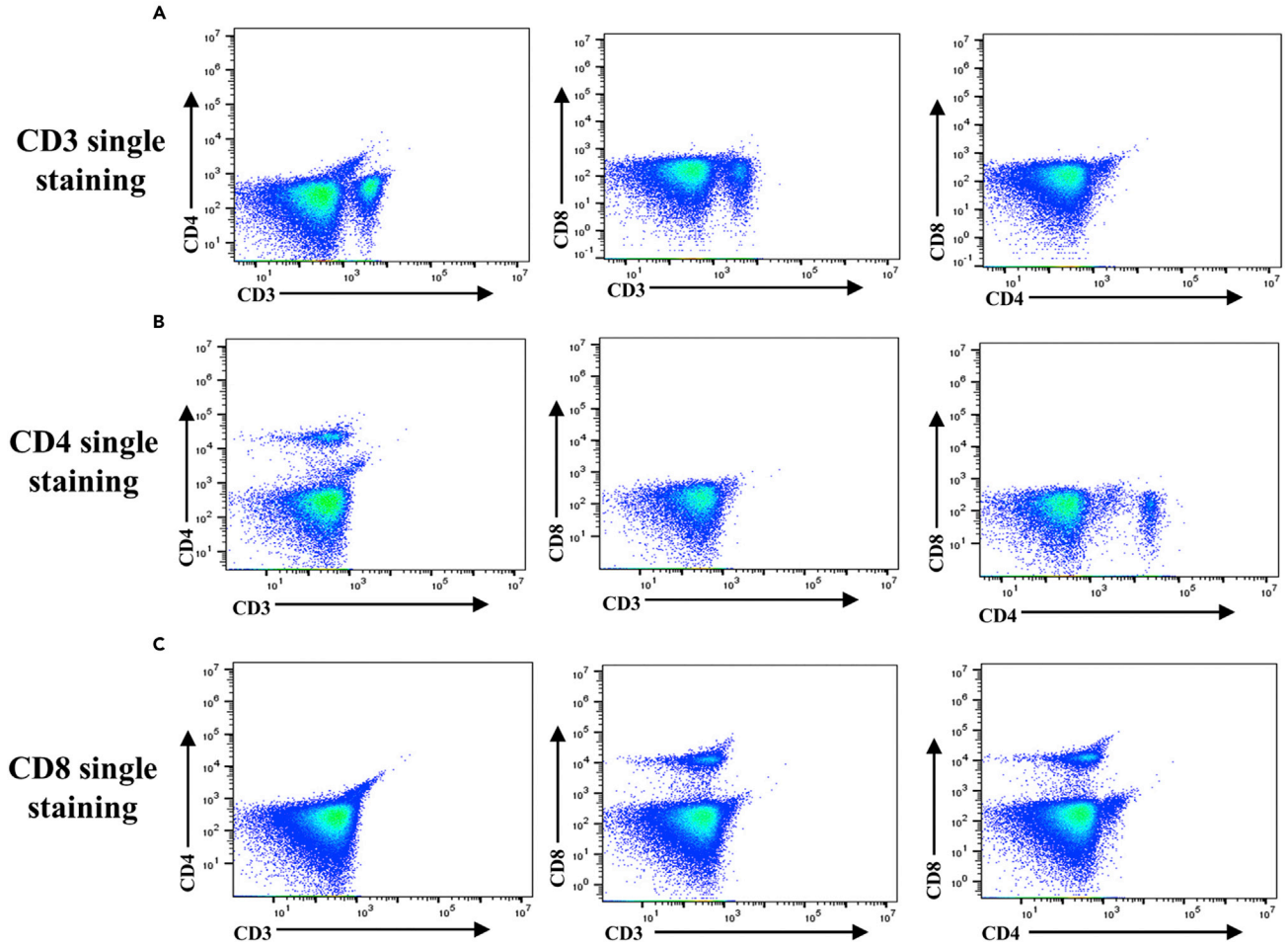


Figure 3. Adjustment of the fluorescent compensation by single staining tube

(A–C) Set CD3 vs CD4, CD3 vs CD8, CD4 vs CD8 gate respectively, adjust fluorescent compensation with single staining tubes of CD3 (A), CD4 (B) and CD8 (C).

Flow cytometry analysis

Timing: about 30 min

50. Filter the single cell suspension with a 200-mesh filter membrane and place them in the flow tubes.
51. Adjust the fluorescence compensation with single staining tube. The operation of adjusting fluorescence compensation is as follows, taking the detection of CD3, CD4 and CD8 indicators as an example.
    a. According to the required staining indicators, make a pair combination and set the X-Y plot on the computer in advance. When the number of indicators is n, the total number of plots is 3×(n-2).
    b. Collect the cells of the single staining tube respectively, and adjust the compensation value, so that there is a detection signal on the staining channel and no signal on other non-staining channels. The compensation plots of each single staining tube are shown in Figure 3.

Note: Fluorescence of all detected single staining samples cannot be in the same fluorescent channel.

52. Analyze cell samples with a flow cytometry. For samples stained only on the cell surface, collect 1×10^5 cells. For the cell samples for detecting intracellular factors, collect 5×10^4 cells.

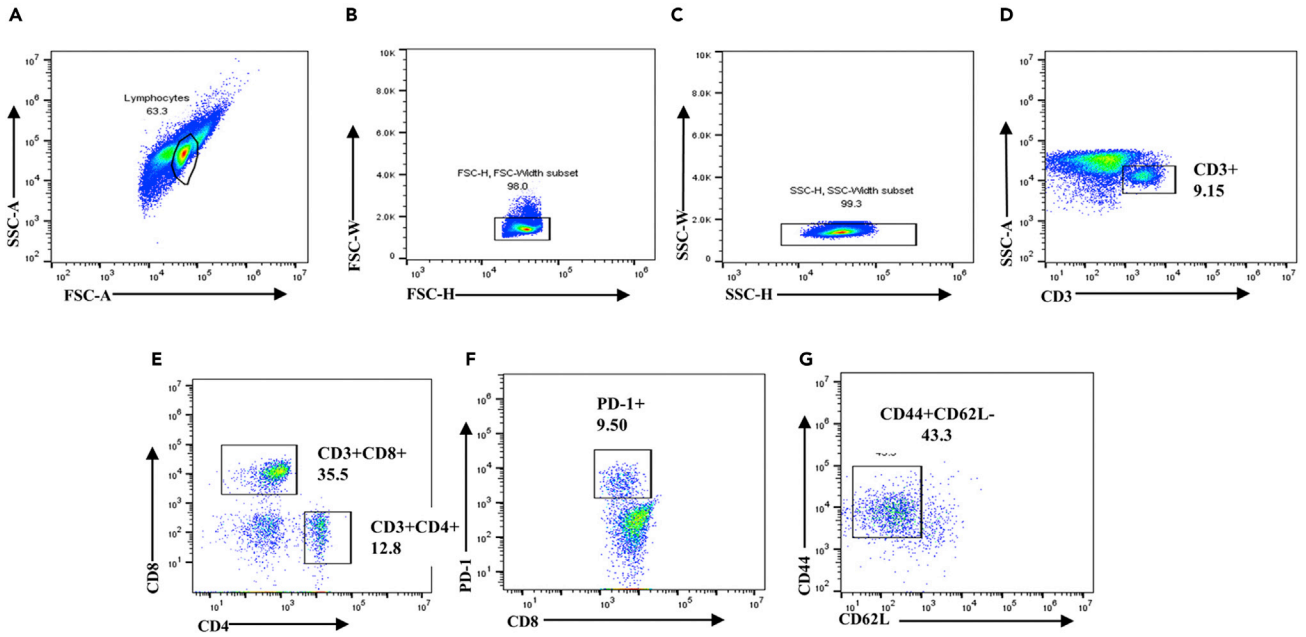


Figure 4. Flow cytometric analysis of the ratio of immune cells infiltrated in tumor

(A) Set the initial FSC vs SSC gate, gating T lymphocytes.

(B and C) Set FSC-H vs FSC-W (B) and SSC-H vs SSC-W gate, removing adherent cells (C).

(D) CD3+ cell gating to identify the total tumor-derived T lymphocytes.

(E) Gating of CD4+ and CD8+ T cells within CD3+ T cell population.

(F) Gating of PD-1+ T cells within CD8+ T cell population.

(G) Gating of CD44+CD62L- T cells within CD8+ T cell population.

Note: The number of cells used for flow cytometric analysis is not fixed. In tumor cells, due to the small proportion of some infiltrating immune cells, the number of collected samples should be expanded in order to be able to detect certain populations.

53. The detailed gating strategy shown as Figures 4 and 5.
    a. Set the initial FSC vs SSC gate to circle the lymphocyte population (Figures 3A and 4A).

Note: In this step, we have carefully circled the main cell cluster, largely excluding the stray cells and dead cells.

    b. Set FSC-H vs FSC-W gate (Figures 3B and 4B) and SSC-H vs SSC-W gate (Figures 3C and 4C) to remove adherent cells.

⚠ CRITICAL: The cell population with constant height (H) and increased width (W) are adherent cells.

    c. Set CD3 vs SSC gate to circle the CD3+ T lymphocytes (Figures 4D and 5D).
    d. Set CD4 vs CD8 gate to circle the CD4+ T lymphocytes and CD8+ T lymphocytes (Figure 4E); Set CD8 vs SSC gate to circle the CD8+ T lymphocytes (Figure 5E).
    e. Gated CD8+ T lymphocytes. Set CD8 vs PD-1 gate to circle CD8+PD-1+ T lymphocytes (Figure 4F); Set CD62L vs CD44 gate to circle CD44+CD62L- T lymphocytes (Figure 4G); Set CD8 vs IFN-γ to circle CD8+PD-1+ T lymphocytes (Figure 5F).

Note: In the process of flow cytometric detection, operate under dark condition.

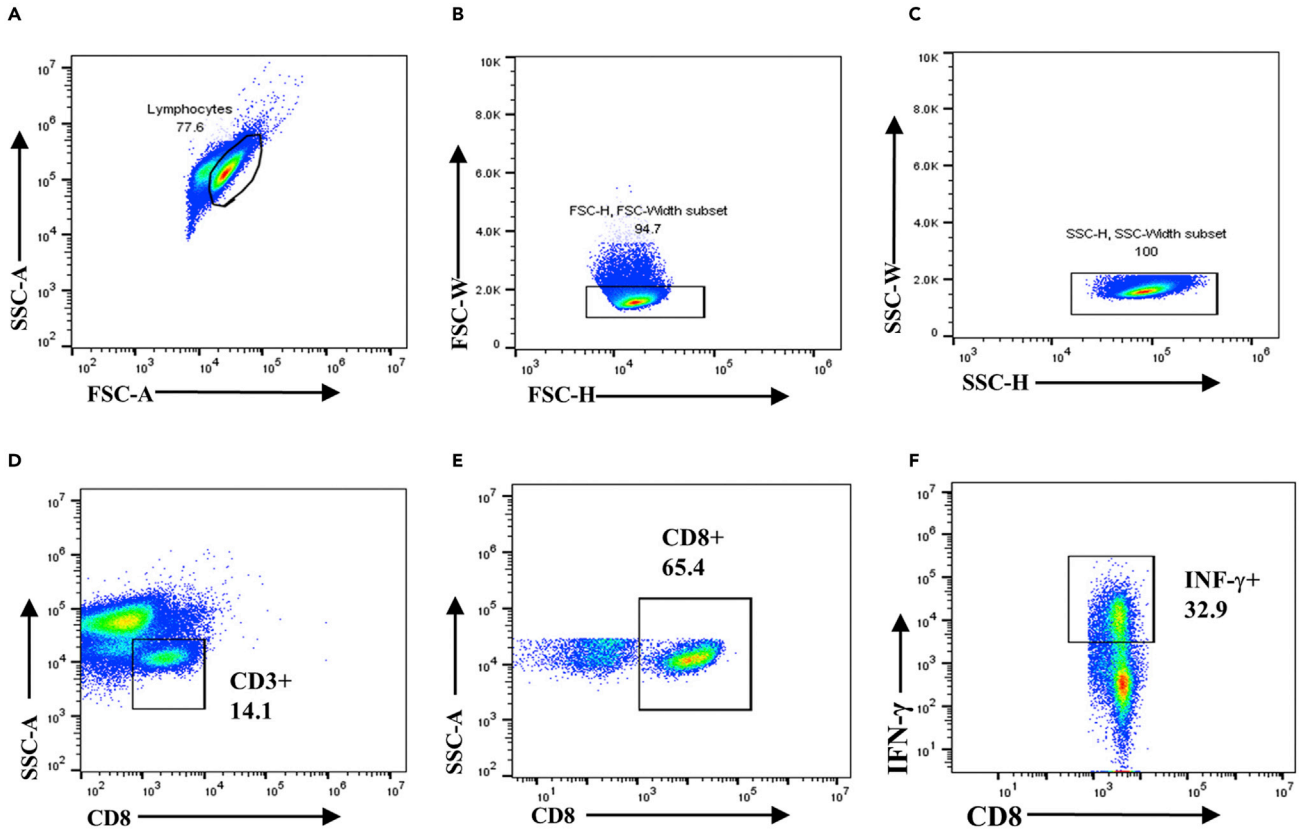


Figure 5. Flow cytometric analysis of the percentage of IFN-γ positive cells in CD8+ T cells

(A) Set the initial FSC vs SSC gate, gating T lymphocytes.

(B and C) Set FSC-H vs FSC-W (B) and SSC-H vs SSC-W gate, removing adherent cells (C).

(D) CD3+ cell gating to identify the total tumor-derived T lymphocytes.

(E) Gating of CD8+ T cells within CD3+ T cell population.

(F) Gating of IFN-γ+ T cells within CD8+ T cell population.

54. Analyze the sorting results with FlowJo software. The analysis results of the proportion of tumor-infiltrated immune cells and the proportion of IFN-γ released by CD8+ T cells are presented in Figures 3 and 4, respectively.

Note: In the process of flow cytometry analysis, operate under dark condition.

EXPECTED OUTCOMES

According to previous research, the fractional irradiation scheme of 8 Gy×3 can effectively induce ATAE.[1,8,9] In this protocol, we provided reliable construction method of primary tumor and abscopal tumor model and fractional irradiation model in mice, as well the detailed dosage regimen of drugs that further affected ATAE effectively.

Based on the protocol of single cell isolation by digesting tumor tissue, the effects of fractionated irradiation and drug treatment on the proportions of infiltrated immune cells in mice xenograft tumors are detected by flow cytometry assay.

LIMITATIONS

One of the limitations is that, in our experiment, we explore the ATAE by constructing the bilateral tumor model rather than the spontaneous metastatic tumor model due to the low success rate in

constructing the model of spontaneous metastasis, which has limitation on the effect of simulating the clinical ATAE. In addition, we only identified the function of CD8+ T cells and the production of cytokines released by CD8+ T cells by labeling surface antigens, but did not sort out the CD8+ T cells infiltrated in the tumors to detect relevant indicators because of the low proportion of infiltrated CD8+ T cells in tumors. Therefore, it is worthwhile to explore more feasible solutions to solve the technical obstacles in future.

TROUBLESHOOTING

Problem 1

During PCR assay, the DNA bands in agarose gel are too weak to be observed.

Potential solution

Improve experimental conditions. Use new primers and store it in a short period if necessary. Increase the amount of cDNA and/or the number of PCR cycles (30–35 cycles) appropriately, or reduce the annealing temperature.

Problem 2

Low tumor formation rate or no tumor formation after tumor cell inoculation.

Potential solution

Insufficient cell viability or low inoculation density will affect the tumor formation efficiency in mice. It's necessary to conduct a pre-experiment to determine the optimal amount of cells that can form tumors in mice in advance. Cells should be inoculated in the shortest time after digestion and place on ice to ensure viability. Some kind of cells are difficult to form tumors, in this case, mix the Matrigel with cell suspension (Matrigel: PBS=1: 1) to improve the tumor formation rate.

Problem 3

The tumor sizes formed by inoculating the same number of tumor cells in the same experimental group of mice are significantly different.

Potential solution

The reason for this difference in tumor size may be that the tumor cells are not evenly mixed during the inoculation, or the tumor cells flow out after the injection resulting in not all of them being inoculated subcutaneously. In this case, gently flick the EP tube and syringe tube wall to mix the cells well before inoculating the cells into mice, and do not mix cells too violently to avoid mechanical damage to cells.

Problem 4

The amount of single cells isolated from tumor tissue is less than expected number.

Potential solution

In the process of isolating single cells from tumor tissue, the tumor tissue should be cut as small as possible so that it can be fully digested by collagenase and DNase. Ensure that the digestion time of collagenase is less than 1.5 h to prevent excessive digestion leading to cell death.

Problem 5

Weak immunofluorescence signals in the process of flow cytometry analysis of cell surface markers.

Potential solution

Increase the corresponding antibody concentration or staining time, or replace the antibody labeling with stronger fluorescence (such as using the fluorescein of phycoerythrin (PE) and allophycocyanin (APC), or apply the antibody produced by different manufacturers.

Problem 6

In the process of cell staining, we carefully circled the main cell cluster, which largely excluded the stray cells and dead cells. Meanwhile, considering that there were many staining indicators in our experimental scheme and the fluorescent signals would interfere with each other, so we did not use the live/dead dyes to stain the cells. But for the rigor of the experiment, live/dead cell staining may necessary, which can eliminate the interference of dead cells.

Potential solution

Select appropriate live/dead dyes according to suitable live/dead dyes according to the principle that the fluorescent channels do not interfere with each other, and stain the samples with live/dead dyes according to the manufacturer's instructions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chunlin Shao, clshao@shum.edu.cn.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets.

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AUTHOR CONTRIBUTIONS

X.Z. prepared all the figures and drafted the manuscript. C.S. conceived, supervised, directed the project, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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