EXPERIMENTAL PROTOCOL: Cell prep and *in vivo* tumor inoculation (subcutaneous)

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Introduction

Subcutaneous inoculation of tumor cells into mice is an essential technique that can be used for multiple applications (tumor responses to treatment -syngeneic or xenograft models-, phenotypic validation of genetic variants *in vivo*, analysis of *in situ* TME, …). This protocol is designed to describe the different steps to prepare cells for a tumor challenge in mice. It is intended to be a general protocol for commonly used syngeneic mouse tumor cell lines. Therefore, it is very important to adjust cell culture and inoculation conditions when necessary.

Tumor growth kinetics and response to treatments (i.e., checkpoint blockade with anti-PD-1 and/or anti-CTLA-4) will vary significantly between models. Cell culture and inoculation skills can impact dramatically the outcome of *in vivo* growing tumors.

All *in vivo* procedures must comply with local regulations and included in an experimental protocol.

Reagents

- Complete cell culture media (RPMI, DMEM + FBS +P/S + …)
- HBSS (PBS can be used as well)
- (*optional*) Matrigel (recommended Corning cat # 356231)
- Ketamine/xylazine (or similar for mouse anesthesia)
- 23G/25G needle and syringes
- Cell counting reagents/materials

Before start

- Mouse and cell numbers should be tailored to your model and the needs of a specific experiment

- When setting up flasks for a challenge, take into account that you will lose cells during cell prep/washing/needle-loading so it is important to seed extra cells so you will have sufficient numbers
- When required, Matrigel (used at 50/50 dilution in HBSS injection solution to help cells engraft, stored in freezer room) must be thawed very carefully on ice and kept on ice at all times. The solution is frozen below 0C and solidifies permanently at room temperature; while it is okay to initially thaw on the bench or in the hood, place in ice well before melted to keep from solidifying.

PROCEDURE

CELL EXPANSION FOR CHALLENGE

1. Thaw cells at least 5-7 days before challenge, depending on specific cell line and selection needs:
    - At least 1 day after thawing: for cells that express vectors (i.e., Cas9, OVA…) containing selection markers, select with the relevant antibiotic for 2-3 days (see Annex I- antibiotic master sheet) to ensure uniform protein expression. For cells expressing multiple selection markers, select with each antibiotic sequentially–do NOT add multiple antibiotics to the same cells simultaneously (the combination could be non-selectively fatal depending on the cell line and specific antibiotics).
2. While selection is happening, start expanding cells into larger/more flasks for challenge day. The following considerations can be used to plan how many cells you will need to culture for the challenge. Be conservative when estimating cell yield from each flask.
    - Numbers of cells injected can vary by experiment. Be sure to account for the number of cells you need when you set up flasks for the challenge. The general guideline is 0.5-1e6 cells/mouse
    - During wash and cell prep steps cells are lost. In order to account for this loss, plan for ~50% overage of the cell number you need based on what you expect to get from that flask. (i.e. if you need 10E6 cells, culture 15E6 cells)
    - Cells are also lost during injection; during challenge cell culture, plan to have overage of cells based on type of mouse used in experiment (~25% more cells than you are planning to inject)
3. It is critical that for experiments where you will be directly comparing two or more cell lines (i.e., WT and KO) that the cells be prepped in exactly the same

way leading up to the challenge. Always count the cells prior to seeding the flasks and seed exactly the same number of cells in fresh TC plastic.

- The final cell passage should be 2-3 days before challenge; at this point you should set up the number of flasks you think you will need taking into account the above considerations. Do not split cells the day before the challenge (check to make sure they're happy and change media if necessary).

DAY OF CHALLENGE: Cell Culture

1. Examine cells under the microscope.
   - Do cells look ready for the challenge? It is always better to identify issues early (e.g. cells look strange, low numbers) before you start.
2. Collect cells and combine all flasks of the same cell line into a labeled 50 mL conical (or more, if needed)
   - Do NOT use 15 mL conicals as the mouth is too narrow for injection needles
3. Spin cells out of trypsin/media for 4-5 min, 350 rcf. Keep cells and HBSS on ice when not spinning to help preserve viability.
4. Aspirate media and resuspend pellet in 1x sterile HBSS (Use a volume equal to or up to 50% less than the volume used when splitting the cells - i.e. 10 mL for a T75 or 20 mL for a T225 would be appropriate)
5. Spin down at 350 rcf (1st wash), aspirate, and resuspend in HBSS.
6. Spin down at 350 rcf (2nd wash)
7. Aspirate HBSS and combine all cells of the same cell line/condition in 1 vessel.
8. Count each cell line at least twice. Average counts, and calculate final volume needed to resuspend cells at desired concentration
   - If live cell counts differ by more than 10%, re-count cells.
   - Injections are usually done with 200 uL/tumor

Cells/tumor	Concentration for challenge
0,5E6	2.5E6 cells/mL
1E6	5E6 cells/mL

9. Spin down at 350 rcf (3rd wash). Resuspend in calculated volume of HBSS.
   - *Optional:* You might need to resuspend in a 50/50 mixture of HBSS/matrigel. First completely resuspend cells in HBSS at 2x (half the

total calculated volume). Then add the remaining volume of matrigel while keeping cells on ice as much as possible; pipette up and down **briefly** to mix.

DAY OF CHALLENGE: Mouse House

Notes:

- If working with immunodeficient mice (i.e. NSG) it is essential to maintain sterility. Follow appropriate guidelines for handling rules, PPE, etc. It is recommended to use individual needles for each animal to minimize contamination (especially with NSG mice)

1. Inject anesthetic soluction (i.e., ketamine/xylazine) into each mouse.
    - Check for dermatitis in B6 mice before injecting -- if mice have discernable derm, do not use them.
    - Randomize female mice between cages for large experiments (do not mix male mice from different cages; they will fight)
    - *NSGs:* Use a fresh needle for each mouse to prevent cross-mouse infection (this could take a while, recommended to prepare material in advance)
2. Once mice are unconscious, shave mouse's flank.
    - Tumors are injected on the right flank for unilateral expts
    - Ensure that mouse's nose is free to breathe in cage (not buried under bedding or cagemates)
3. Inject 200 uL tumor cell solution subcutaneously into mouse's flank using 23-25 gauge needle.
    - *NSGs:* use a fresh needle for each mouse
    - *10x/TIL analysis experiments:* inject cells on the belly or back of the mouse to avoid lymph nodes. Insert needle parallel to belly directly SQ. Be careful not to inject IP (keep needle parallel to the belly at all times).
    - Remove needle very slowly to ensure that cells in matrigel/HBSS do not leak out.
4. After mice are injected, follow experimental protocol as required (i.e., place cages on heating pads, fill out experimental cards, …).

Annex I: antibiotic master sheet

Cell Line Name	Media Used	Approximate time to trypsinize	Approximate time between two 1:10 passages (in days)	Blasticidin *	Hygromycin *	Puromycin *	Special considerations	Genetic background
B16	DMEM	3-5 minutes	2	5 ug/mL	125 ug/mL	2 ug/mL		C57BL6
CT26	RPMI	3-5 minutes	2-3	10 ug/mL	500 ug/mL	20 ug/mL		BALB/c
MC38	DMEM	3-5 minutes		5 ug/mL	625 ug/mL	4 ug/mL		C57BL6
KPC	DMEM	5-10 minutes in incubator	2	10 ug/mL	500 ug/mL	10 ug/mL		C57BL6
KP1.9	DMEM	10-15 minutes in incubator	2	5 ug/mL	125 ug/mL	2 ug/mL		C57BL6
LLC	DMEM	2-3 minutes	2	5 ug/mL	250 ug/mL	2 ug/mL	Cells might have a mixed phenotype, so they can look round/semi-adherent. This is normal, although ours are mostly adherent	C57BL6
A20	RPMI	Suspension cell line; do not trypsinize	2	5 ug/mL	125 ug/mL	2 ug/mL	Difficult to transduce with Lenti, requires additional optimization.	BALB/c
4T1	RPMI	10-15 minutes in incubator	2-4	20 ug/mL	250 ug/mL	4 ug/mL		BALB/c
ID8	DMEM		1-2	25 ug/mL			Not tumorigenic (requires additional optimization) Flasks must be washed with 1X	C57BL6
Renca	RPMI	5 minutes	2-3	5 ug/mL	625 ug/mL	2 ug/mL	PBS between passages and	BALB/c
D4m3a	DMEM	3-5 minutes	2-3	5 ug/mL	250 ug/mL	2 ug/mL		C57BL6
Yummer1.7	DMEM +1:100 NEAA	2-3 minutes 5-10 minutes in	2-4	5 ug/mL	250 ug/mL	4 ug/mL	~~Very difficult to thaw,~~ recommend freezing in Bambanker freezing media with 5E6 per vial and spinning out of freezing media when thawing. Plan ahead when thawing, take ~1 week to become fully confluent	C57BL6 (male only)
Panc02	DMEM	incubator	2-3	10 ug/mL	625 ug/mL	6 ug/mL		C57BL6

*Concentrations of selection drugs are suggestive and should be empirically determined in each lab.